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PROMOTING NITROGEN EFFICIENCY IN AQUACULTURE FISH TO ENSURE A MORE SUSTAINABLE PRODUCTION

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Declaração de autoria do trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

À Leonor

“És tu a Primavera que eu esperava,

A vida multiplicada e brilhante,

Em que é pleno e perfeito cada instante.”

Sophia de Mello Breyner Andresen

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Teodósio, R., Engrola, S., Cabano, M., Colen, R., Masagounder, K., Aragão, C., 2021. Metabolic and nutritional responses of Nile tilapia juveniles to dietary methionine sources. Br. J. Nutr., in press. ***doi: 10.1017/S0007114521001008***

Teodósio, R., Aragão, C., Colen, R., Carrilho, R., Dias, J., Engrola, S., 2021. A nutritional strategy to promote gilthead seabream performance under low temperatures. Aquaculture 537, 736494. ***doi: 10.1016/j.aquaculture.2021.736494***

Two publications will be submitted to international peer-reviewed journals as follows:

Teodósio, R., Aragão, C., Conceição, L.E.C., Dias, J., Engrola, S. Metabolic fate is defined by amino acid nature in gilthead seabream (*Sparus aurata*) fed different diet formulations. **To be submitted to Amino Acids.**

Teodósio, R., Aragão, C., Conceição, L.E.C., Dias, J., Engrola, S. Amino acid metabolism in gilthead seabream is differently affected by the dietary protein to energy ratios. **To be submitted to Aquaculture.**

SUMMARY

The sustainability of the aquaculture industry depends on formulating diets that promote nitrogen retention and maximise fish growth performance. This Thesis was focused on the optimisation of environmentally sustainable and cost-effective feeds for two fish species relevant to the World and European aquaculture: Nile tilapia (*Oreochromis niloticus*) and gilthead seabream (*Sparus aurata*). **Chapter II** aimed to reduce protein inclusion levels in plant-based diets for juvenile tilapia, through adequate amino acid supplementation, to minimise diet environmental impact and increase biological efficiency. Results showed that dietary protein content may be reduced to 30% without hindering growth performance. Additionally, amino acid supplementation of a lower protein diet improved protein utilisation and decreased amino acid catabolism, leading to lower nitrogen losses. In **Chapter III**, the bioavailability and metabolic fate of ketogenic and glucogenic amino acids in juvenile seabream fed plant-based diets with distinct protein and/or lipid content was evaluated. Results established that the metabolic fate of amino acids was mainly affected by their nature rather than the diet formulations. Similar results were obtained in **Chapter IV** in seabream fed diets differing in the inclusion of lipids with distinct protein to energy ratios. Findings from **Chapters III** and **IV** showed that in seabream juveniles methionine was preferentially retained in the muscle while lysine was catabolised. In **Chapter V**, the influence of different methionine sources on metabolism and growth of tilapia was investigated. Fish fed a diet supplemented with DL-methionine showed improved growth compared with fish fed a non-supplemented diet, while dietary supplementation with methyl hydroxyl analogue resulted in intermediate values. The impact on fish growth was probably due to the distinct metabolic pathways used for converting methionine, which were different between sources. **Chapter VI** evaluated growth performance, diet digestibility and nitrogen balance in seabream reared under low temperatures. The supplementation of feed additives in diets for seabream juveniles enabled the decrease of protein and fishmeal contents by increasing diet digestibility and lowering nitrogen faecal losses. This Thesis generated relevant knowledge on nutritional strategies to promote nitrogen retention and maximise fish growth, towards a more sustainable aquaculture.

Keywords: Fish nutrition; Aquaculture sustainability; Nitrogen retention; Protein utilisation; Amino acid bioavailability; Dietary supplementation.

RESUMO

A sustentabilidade da aquacultura depende da formulação de dietas que promovam a retenção de azoto e potenciem a performance de crescimento dos peixes. Esta Tese teve como principal objetivo a otimização de dietas economicamente viáveis e ambientalmente sustentáveis para duas espécies de peixes relevantes no panorama mundial e europeu da indústria da aquacultura: a tilápia do Nilo (*Oreochromis niloticus*) e a dourada (*Sparus aurata*). No **Capítulo II**, através da suplementação adequada de aminoácidos em dietas vegetais para juvenis de tilápia, pretendeu-se reduzir os níveis de inclusão de proteína de forma a minimizar o impacto ambiental e aumentar a eficiência biológica. Demonstrou-se que o conteúdo proteico das dietas pode ser reduzido para 30% sem prejudicar o crescimento dos peixes. Além disso, a suplementação com aminoácidos de uma dieta com um menor conteúdo de proteína melhorou a utilização deste nutriente e diminuiu o catabolismo dos aminoácidos, resultando em menores perdas de azoto para o ambiente. No **Capítulo III** foi avaliada a biodisponibilidade, assim como o destino metabólico de aminoácidos cetogénicos e glucogénicos em juvenis de dourada alimentados com dietas vegetais com diferentes níveis de proteína e/ou lípidos. A utilização metabólica dos aminoácidos foi maioritariamente influenciada pela sua natureza cetogénica ou glucogénica e não pela formulação das dietas. No **Capítulo IV** os resultados foram semelhantes em douradas alimentadas com dietas com diferentes níveis de inclusão de lípidos, diferindo no rácio de proteína e energia. Os resultados obtidos nos **Capítulos III** e **IV** demonstraram que, em juvenis de dourada, a metionina foi preferencialmente retida no músculo enquanto a lisina foi catabolizada. No **Capítulo V** foi estudada a influência de diferentes fontes de metionina no metabolismo e crescimento de tilápias. O crescimento de peixes alimentados com uma dieta suplementada com DL-metionina foi superior ao observado em peixes alimentados com uma dieta não suplementada, enquanto os peixes alimentados com uma dieta suplementada com um análogo da metionina ("methyl hydroxyl analogue") tiveram um crescimento intermédio. Este impacto na performance de crescimento dos peixes poderá ser devido ao facto das diferentes fontes de metionina seguirem vias metabólicas distintas na sua conversão. No **Capítulo VI** foi avaliado se dietas formuladas com aditivos alimentares poderiam ajudar os peixes a lidar com condições não ótimas de cultivo, neste caso baixa temperatura de água. Foram avaliados parâmetros de crescimento, digestibilidade de dietas e balanço de azoto em douradas. A suplementação com aditivos alimentares tornou possível reduzir a proteína e a farinha de peixe das dietas, devido a um aumento da digestibilidade e a um decréscimo nas perdas fecais de azoto. Esta Tese permitiu aumentar o conhecimento sobre como diferentes estratégias nutricionais ajudam a promover a retenção de azoto e a potenciar o crescimento dos peixes, contribuindo para o desenvolvimento de uma aquacultura mais sustentável.

Palavras-chave: Nutrição de peixes; Sustentabilidade da aquacultura; Retenção de azoto; Utilização de proteína; Biodisponibilidade de aminoácidos; Suplementação de dietas.

Chapter I

General Introduction

1.1. AQUACULTURE – AN OVERVIEW

Fish is an exceptional source of protein, fatty acids and essential micronutrients required for human consumption. Fish demand is increasing globally due to a combination of factors, among them the consumer awareness of its nutritional value and health benefits, the rise in populations income, and technological developments in the production and supply chains. With the World's population expected to exceed 9 billion people by 2050 (United Nations estimates), and the stagnation of capture fisheries for the last decades as shown in Figure 1.1, aquaculture plays an important role in fulfilling this demand.

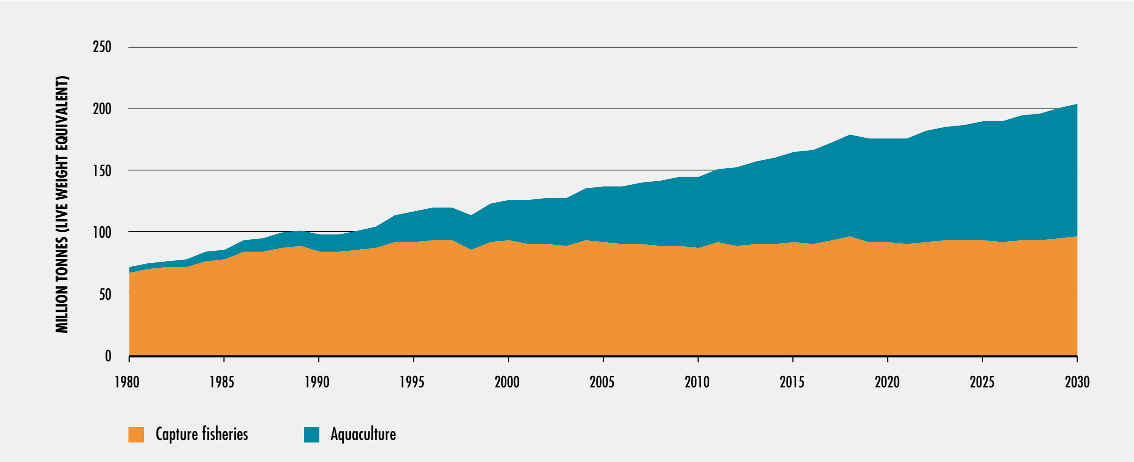

Figure 1.1 World capture fisheries and aquaculture production from 1980 to 2030. *Source: FAO (2020a).*

In 2018, total aquaculture production reached 114.5 million tonnes worldwide, of which 82.1 million tonnes derived from farming of aquatic animals, representing 46% of the total fish[1] production (capture fisheries and aquaculture) and 52% of the total supply of fish for human consumption (FAO, 2020a). Nevertheless, by 2030 this sector is expected to surpass total capture fisheries and to contribute with 59% to the global food fish consumption, as illustrated in Figures 1.1 and 1.2.

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[1] For simplification, the term "fish" refers to fish, crustaceans, molluscs and other aquatic animals, but excludes aquatic mammals, reptiles, seaweeds and other aquatic plants.

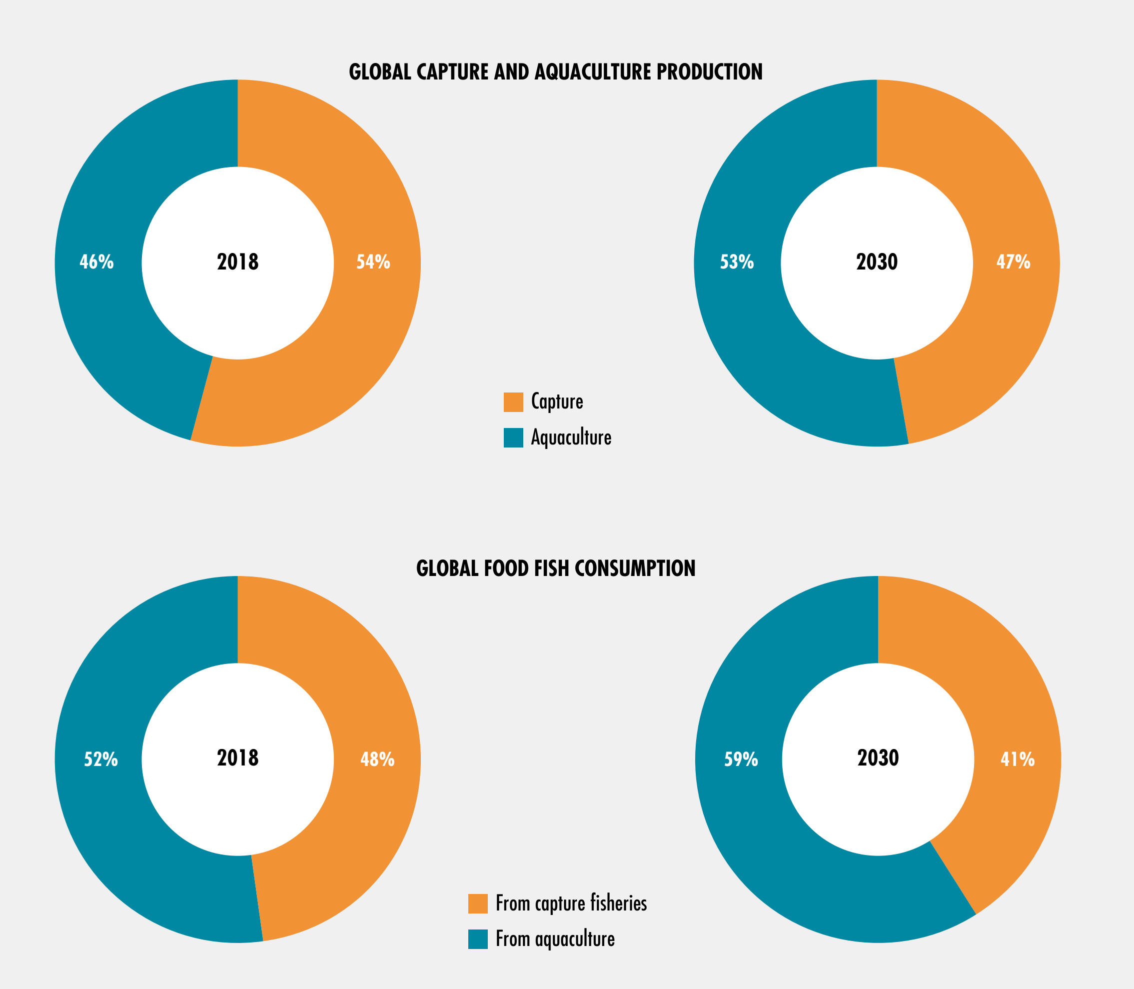

Figure 1.2 Increasing role of aquaculture. *Source: FAO (2020a).*

Although the aquaculture sector remains one of the most thriving animal production industries, its expansion has suffered a deceleration over the last years, mainly caused by the slowdown in China's aquaculture production due to governmental policies introduced in 2016. The average annual growth rate of the sector was 5% from 2011 to 2015 but has declined to 3.6% during 2016 – 2018 (FAO, 2020b). Nevertheless, China remained the largest fish producer in the World, contributing with over 57% to the global aquaculture production in 2018. Excluding China, Asia is still the greatest producer with 34% share to global fish farming, followed by the Americas (14%), Europe (10%), Africa (7%) and finally Oceania (1%). From the top ten aquaculture producers only Egypt, Norway and Chile, are not located in the Asian continent (Table 1.1).

Table 1.1 World total and top ten aquaculture producers (excluding aquatic plants and non-food products) in 2018.

Country	Quantity (million tonnes)	Value (billion USD)
World total	82.1	250.1
China	47.6	145.0
India	7.1	13.2
Indonesia	5.4	12.0
Vietnam	4.1	14.5
Bangladesh	2.4	5.9
Egypt	1.6	1.5
Norway	1.4	8.3
Chile	1.3	10.4
Myanmar	1.1	1.5
Thailand	0.9	2.7

Source: FAO (2020b).

In 2019, European aquaculture (European Union - EU and non-EU countries) experienced a greater annual growth rate of 7% compared with the World industry (FEAP, 2020). Norway leads the European industry, dominated by the production of Atlantic salmon (*Salmo salar*), which represented almost 65% of the total European fish production in 2019. Turkey and Greece are the second and fourth top fish producers in Europe, mainly farming marine species such as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). United Kingdom is ranked at number three, predominantly cultivating Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*).

1.2. TOWARDS A SUSTAINABLE AQUACULTURE

In 2015, the United Nations (UN) endorsed what is known as the 2030 Agenda for Sustainable Development and committed to its 17 Sustainable Development Goals (SDGs) (United Nations General Assembly, 2015). In 2018, the Food and Agriculture Organization of the United Nations (FAO) developed an action plan to achieve the SDGs in the food and agriculture sector, in which aquaculture is included (FAO, 2018c, d). The strategy is based on five principles that balance the social, economic, and environmental dimensions of sustainability. The five key principles are: 1) increase productivity, employment, and value addition in food systems; 2) protect and enhance natural resources; 3) improve livelihoods and foster inclusive economic growth; 4) enhance the resilience of people, communities, and ecosystems; and 5) adapt governance to new challenges. Table 1.2 summarises the actions proposed by FAO for the sustainable development of aquaculture based on these five principles.

Among the SDGs, six are closely associated with the aquaculture industry (Figure 1.3). SDG 2 – achieve food security and improved nutrition; SDG 12 – ensure sustainable consumption and production patterns; SDG 13 – combat climate change and its impacts; SDG 14 – sustainably use the oceans and marine resources; SDG 15 – promote sustainable use of terrestrial ecosystems; and SDG 17 – implement and revitalise the global partnership for sustainable development.

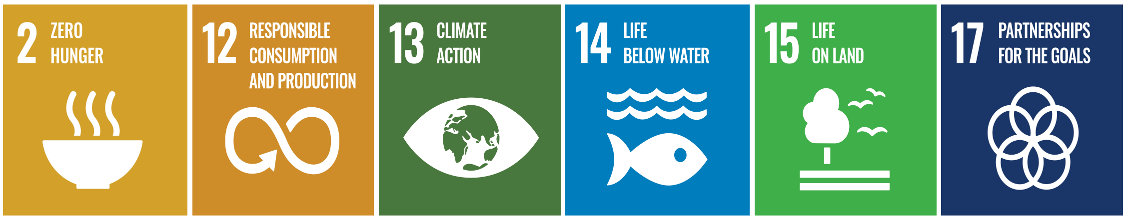

Figure 1.3 Aquaculture in the 2030 Agenda for Sustainable Development. The United Nations Sustainable Development Goals (SDGs) associated with the aquaculture industry. *Adapted from FAO (2018c, d).*

Table 1.2 Key policies and practices for engaging aquaculture in the 2030 Agenda.

Increase productivity, employment and value addition in food systems	Protect and enhance natural resources	Improve livelihoods and foster inclusive economic growth	Enhance the resilience of people, communities and ecosystems	Adapt governance to new challenges
Aquafeed management	Conserve aquatic genetic resources	Aquaculture business-model, especially for small holder producers	Assess risks in aquaculture (pathogens, food safety, ecological, environmental, including climate change), genetic, social and financial)	Compliance with international treaties, standards, agreements on sustainable aquaculture, animal health, food safety
Integrated multitrophic aquaculture	Promote aquaculture certification for environmental protection	Aquaculture certification including gender considerations	Set early warning, preparedness, surveillance systems and contingency plans for aquatic emergencies	Voluntary adoption of BMPs, GAPs
Robust biosecurity/aquatic animal health	Ensure biosecurity: pathogens, escapees, use of veterinary drugs, invasive species, biodiversity	Cooperative marketing	Implementing the Ecosystem Approach to Aquaculture (EAA)	Contribution and impact assessments
Use of best management practices (BMPs), good aquaculture practices (GAPs), codes	Use integrated aquaculture-agriculture systems	Mainstream aquaculture into rural development processes		Implementing the Ecosystem Approach to Aquaculture (EAA)
Domestication of aquaculture species	Implementing the Ecosystem Approach to Aquaculture (EAA)	Technological, trade, institutional, infrastructure, capacity development, investment and other support services		
Aquaculture certification for animal health and welfare, and food safety		Aquaculture and nutrition programs		
Implementing the Ecosystem Approach to Aquaculture (EAA)		Implementing the Ecosystem Approach to Aquaculture (EAA)		

Source: FAO (2018d).

The concept of “Sustainable Food Systems” was put forward as a “system that delivers food security and nutrition for all in such a way that the economic, social and environmental bases to generate food security and nutrition for future generations are not compromised” (FAO, 2018b). At the European level and in line with the European Green Deal, the Commission has issued the “Farm to Fork Strategy” with the purpose of building the food chain that works for consumers, producers, climate and the environment (European Commission, 2020). Recently, the strategic guidelines for a more sustainable and competitive EU aquaculture for the period 2021 to 2030 were published (European Commission, 2021). These guidelines aim on helping to build a competitive and resilient industry to become a global reference for sustainability by ensuring the supply of nutritious and healthy food, reducing the EU’s dependency on seafood imports, and creating economic opportunities and jobs. Nevertheless, both FAO and EU recognise that aquafeed management and the use of sustainable feeds to reduce the carbon footprint of the sector are part of the solution towards a more sustainable aquaculture.

1.2.1. Sustainable fish feeds

The need to reduce the dependency of the aquafeed sector on finite marine ingredients, particularly fishmeal and fish oil, has been driving the industry in searching for new raw materials (Matos et al., 2017). This is especially true for protein sources, not only because most of the cost associated with fish diets derives from protein (Kim et al., 2019), but also because the efficiency of fish in using protein dictates growth and nitrogen outputs to the environment.

In the last decades, research efforts were mainly channelled towards substituting marine ingredients by terrestrial plants (Barrows et al., 2008; Gatlin et al., 2007; Ogello et al., 2014; Ytrestøyl et al., 2015). The main moral glitch is that terrestrial crops used to produce fish diets could be directly used for human consumption. Furthermore, agriculture has its own sustainability issues such as freshwater use or deforestation, and in a scenario of climate change, the consistency of harvests can greatly influence the availability, quality and price of the ingredients (Colombo and Turchini, 2021). Nevertheless, diversification is desirable and new plant ingredients, especially by-products from other activities, are constantly emerging and being evaluated. In 2013, the use of terrestrial processed animal proteins (PAP) from non-ruminant origin such as meat, blood, feather and poultry by-product meals, was reinstated in the EU (European Commission, 2013). Recent studies have pointed towards the use of PAP as a good alternative to fishmeal due to their protein content, digestibility, and palatability

(Aragão et al., 2020; Campos et al., 2017; Davies et al., 2019; Sabbagh et al., 2019). Since PAP are by-products of processing plants and slaughterhouses, they are an environmentally sustainable and cost-effective alternative to fishmeal (Jedrejek et al., 2016). Fisheries and aquaculture by-products are also a practical option to substitute fishmeal from wild-caught fish (Stevens et al., 2018). Currently, it is estimated that approximately 25 – 35% of fishmeal and fish oil is produced from by-products of fish processing, which previously were discarded or used directly as feed in silage and in fertilizers (FAO, 2020a; Stevens et al., 2018; Ytrestøyl et al., 2015). These by-products are trimmings (viscera, heads, skin, bones, and blood) after industrial processing of fish for human consumption. Although with a lower protein content than fishmeal from wild captures, these by-products have a very good amino acid and essential fatty acid profile (Hua et al., 2019). Emerging protein sources such as insect meal, macro- and microalgae, and single-cell organisms (*e.g.* yeasts) present encouraging results, yet additional research is needed to achieve the desirable outcomes. The development and optimisation of alternative protein sources for aquafeeds so that environmental sustainability of the aquaculture industry is secured should be considered; however, consumer acceptance, which varies among the different raw materials and in different regions of the World, mainly due to cultural reasons, should also be taken into consideration (Hua et al., 2019).

1.3. NUTRITIONAL STRATEGIES TO PROMOTE NITROGEN RETENTION

The main goal of the aquaculture industry is to maximise production in a cost-effective way. However, this should not be achieved disregarding the associated environmental and social issues of the process. With an increase in World's population, the demand for more nutritious animal protein is also rising. Improving animal performance and health through the optimisation of diets that enhance feed efficiency and promote nitrogen retention is vital to reduce production costs and environmental impacts. No single approach presents the solution for this subject, and multiple strategies that are designed according to fish species, age, size, nutrient requirements, as well as environmental and rearing conditions, should be considered.

The surge of alternative ingredients in diet formulations requires additional attention to the quantitative and qualitative amino acid profiles, which determine protein quality. Dietary protein content may be reduced up to a certain level when dietary amino acid profile is correctly balanced. Several studies have demonstrated the benefits in supplementing diets with crystalline amino acids so that at an effective reduction in dietary protein levels is accomplished without compromising fish growth (Gaylord and Barrows, 2009; Gan et al., 2012).

Maximisation of fish growth requires that amino acids are available in tissues at an optimum ratio, as imbalances will lead to amino acid losses for energy purposes rather than for protein synthesis (Halver and Hardy, 2003). In contrast, optimisation of dietary protein utilisation with a decrease in amino acid catabolism may lead to increased nitrogen retention which may be translated into enhanced growth and lower nitrogen outputs to the environment (Bureau and Hua, 2010). The effects of reducing the dietary protein content through adequate amino acid supplementation will be discussed in Chapter II.

This supplementation is dependent on the fish species and developmental stage and is affected by diet formulation and nutritional composition. Various studies have demonstrated that fish are able to control their amino acid metabolism by regulating the differential use of individual amino acids for retention and catabolism (Aragão et al., 2004; Saavedra et al., 2008a, 2008b). In order to formulate balanced diets that allow fish to reach their full growth potential, it is important to understand how the bioavailability and metabolic fate of amino acids is affected by different dietary formulations, to ensure an optimised utilisation of the feed. This subject will be discussed in detail in Chapters III and IV.

Dietary protein utilisation is determined by numerous factors, among which the digestibility of ingredients, dietary protein content, amino acid profile and protein to energy ratio (P/E) (Halver and Hardy, 2003). Sparing of protein by increasing dietary lipid, and consequently energy content, has been reported for several species resulting in improved growth performance and protein efficiency (Fernandes et al., 2016; Li et al., 2012; Ma et al., 2020; Santinha et al., 1999; Vergara et al., 1996a). Although this protein-sparing effect has been clearly demonstrated in salmonids (Cho and Bureau, 2001), other fish with a lower ability to digest dietary lipids were shown to be unaffected or negatively influenced by the decrease of the dietary P/E ratios under the experimental conditions tested (Borges et al., 2009; Haidar et al., 2018; Ozório et al., 2006). It is fundamental not only to optimise the dietary P/E ratios according to species and farming conditions, but also to consider the bioavailability and metabolism of individual dietary amino acids so that protein is used more efficiently.

The transition from fishmeal-based diets to more sustainable formulations may depend on incorporating mixtures of alternative proteins that complement one another to compensate nutrient imbalances (Oliva-Teles et al., 2015). Currently, soybean meal is the major source of plant protein in aquafeeds and its inclusion in diets has been successful in several species (Carter and Hauler, 2000; Glencross et al., 2004; Nguyen et al., 2009). This ingredient presents a high protein content and relatively well-balanced amino acid profile, although limited in methionine. Therefore, diets require supplementation with this indispensable amino acid to fulfil fish requirements (Gatlin et al., 2007). Synthetic sources of methionine, such as L-methionine, DL-methionine (DL-Met) and methionine hydroxyl analogue (MHA; DL-2-hydroxy-4-methylthiobutyrate and its calcium salt), are commonly added to fish diets to overcome imbalances. However, the biological efficiency of these methionine sources may differ due to structural dissimilarities that may be translated into differences in absorption, transport and metabolism (Goff and Gatlin, 2004; Kim et al., 1992; Powell et al., 2017; Robinson et al., 1978; Sveier et al., 2001). The efficiency of supplemental amino acids from different sources should be considered when formulating diets and information on the effects in metabolism, feed utilisation and growth are essential to assure optimum growth performance as will be seen in Chapter V.

Throughout the production cycle, fish are subjected to a series of potentially stressful events like transport, manipulation for routine procedures (*e.g.* sorting, vaccination) or sub-optimal environmental conditions. Feeding fish with high-quality diets containing high levels of marine-derived protein sources and nutritional supplements revealed to counteract the negative effects of exposure to low temperatures (Richard et al., 2016; Schrama et al., 2017; Silva et al., 2014). However, such an approach fails to follow both economic and environmental sustainability principles. One viable strategy is to formulate diets that reduce dietary protein content and replace marine ingredients such as fishmeal, by ingredients that are eco-friendly but also highly digestible and able to sustain fish growth. The incorporation of feed additives in diets may help fish to sustain growth even under adverse conditions. Rather than providing additional nutrients, feed additives are compounds that may act indirectly as growth promoters by improving feed intake, diet digestibility, and immune system, by reducing oxidative stress or enhancing resistance to pathogens, among other functions (Encarnação, 2016). Some amino acids are added to aquafeeds as functional molecules to regulate key metabolic pathways that are crucial for maintenance, growth or immunity (Wu, 2009). For instance, betaine may be added as a phagostimulant to increase feed intake (Dias et al., 1997; Lim et al., 2015; Xue and Cui, 2001) and taurine is frequently supplemented in taurine-deficient diets, such as plant-based ones, due to its role in lipid digestion, bile acid conjugation and antioxidant defence (Salze and Davis, 2015), and as an attractant and feed stimulant (Chatzifotis et al., 2009). This topic will be addressed in more detail in Chapter VI.

The aforementioned nutritional strategies are valuable approaches in promoting nitrogen retention in farmed fish under environmentally sustainable principles.

1.4. AQUACULTURE AND NUTRITION OF THE MODEL SPECIES

The development of nutritional strategies to promote nitrogen efficiency should be specific for the fish species and consider its nutritional requirements and rearing conditions. Two fish species with high importance for the aquaculture industry were used in this PhD thesis: a freshwater species – Nile tilapia (*Oreochromis niloticus*), and a marine species – gilthead seabream (*Sparus aurata*).

1.4.1. Nile tilapia

Nile tilapia (*Oreochromis niloticus*) is a freshwater fish belonging to the family Cichlidae. Although native to the central and eastern water bodies of Africa, such as Lake Tanganyika and upper Nile basin (Philippart and Ruwet, 1982), during the second half of the 20th century, this species was introduced in many tropical, subtropical and temperate areas of the world as a promising fish for aquaculture (El-Sayed, 2006).

1.4.1.1. Tilapia production

On a global scale, tilapias are the second most cultivated finfish group, with Nile tilapia accounting for 8.3% of total finfish produced in 2018 (FAO, 2020a). This value is probably underestimated since countries such as Bangladesh, India and Pakistan are not included in FAO production statistics. Asian countries lead tilapia farming, followed by Egypt, but its culture is rising in other parts of the world such as Brazil and African countries.

Traditionally, tilapias were farmed extensively and semi-intensively in fertilised earthen ponds (El-Sayed, 2006). However, the global expansion of tilapia production, due to their robustness, disease resistance, and adaptability to a wide range of environmental conditions, high stocking densities and handling procedures, has gradually shifted tilapia culture to more intensive production systems (El-Sayed, 2006; Ng and Romano, 2013). Intensive farming is implemented in a variety of systems, among which the traditional earthen ponds, cages, tanks, raceways, and recirculating aquaculture systems (RAS) (El-Sayed, 2006). Production of all-male populations are favoured since females show lower growth rates, even before sexual maturation (Toguyeni et al., 2002). In contrast, all-male batches are reported to present high growth rates and feed utilisation, high environmental resilience, lower aggression, reduced size variance at harvest and increased resistance to stress and disease (El-Sayed, 2006).

1.4.1.2. Nutrition of Nile tilapia

In the last decades, feeding and nutrition of tilapia has become more pertinent with the intensification of production, and research has devoted great efforts in the optimisation of extruded diets to farm tilapia under optimum conditions of growth and feed utilisation. Commercial feed account for 50% of the total production costs with protein being the most expensive nutrient in the diet (Kim et al., 2019). Optimal protein levels for Nile tilapia diets have been widely studied and, as for other fish species, are dependent on fish size and age, diet digestibility and amino acid profile (Ng and Romano, 2013). While Nile tilapia fry presented optimal growth when fed diets containing 45% crude protein (CP), fingerlings and juveniles obtained better growth performances with 35% CP diets, and adults with 25% CP diets (Abdel-Tawwab et al., 2010; El-Saidy and Gaber, 2005). Nevertheless, feed costs should be considered when formulating diets since tilapias are usually produced and consumed in low-income countries.

Traditionally, commercial diets for Nile tilapia include on average 2% of fishmeal (FAO, 2018a). Fishmeal is considered a reference protein in fish diets, including diets for omnivorous species like tilapia, since it presents a high content of protein with a balanced amino acid profile, it is highly palatable, and it is a source of essential fatty acids, minerals and vitamins (Turchini et al., 2019). The replacement of fishmeal by more economic and/or environmental friendly protein sources in diets for Nile tilapia and its hybrids has been the focus of numerous studies, which demonstrated that the success of this replacement is mainly dependent on a dietary balanced amino acid profile (Figueiredo-Silva et al., 2015; Furuya et al., 2004; Suloma et al., 2014; Trosvik et al., 2012). Tilapia diets usually incorporate a significant amount of terrestrial plant-proteins such as soybean, cottonseed or rapeseed meals (Montoya-Camacho, et al. 2019; Ng and Romano, 2013). Soybean meal is the most widely used plant-protein source and it has been successfully incorporated in tilapia diets to fully or partially substitute fishmeal (El-Saidy and Gaber, 2002; Nguyen et al., 2009). This plant-protein source has a high protein content and digestibility as well as a balanced amino acid composition, making it a viable alternative to fishmeal (Guimarães et al., 2008). The economic and environmental implications in the utilisation of fishmeal and other protein sources in diet formulation requires additional attention and will be addressed in Chapter VI.

Tilapia feeds contain an average of 5% dietary lipids, although studies using levels of 10-15% have shown to improve growth and fillet quality (Ng and Romano, 2013). Deficient or excessive dietary lipid inclusion should be avoided since imbalances can lead to reduced growth or produce excessively fatty fish. Fish oil, included in diets not only as a source of highly digestible energy and essential fatty acids but also for palatability, has been substituted either by single or blends of vegetable oils without negatively affecting growth (Mulligan and Trushenski, 2013; Trushenski et al., 2009; Turchini et al., 2009). Being a warm water omnivorous fish, tilapias are able to utilise dietary carbohydrates with some efficiency, depending on the complexity of the molecule. Carbohydrates are required in fish feeds for binding and stability of the pellets and can also be included as an inexpensive source of energy, sparing dietary protein (Maas et al., 2020).

1.4.2. Gilthead seabream

Gilthead seabream (*Sparus aurata*) is a marine fish that belongs to the Sparidae family. It is geographically distributed throughout the Mediterranean Sea and in the Northeast Atlantic Ocean, from the British Isles to Cape Verde. This species inhabits coastal environments, from seagrass beds, rocky and sandy floors, to surf zones, often entering brackish waters (Basurco et al., 2011).

1.4.2.1. Gilthead seabream production

Gilthead seabream is a highly valuable species, captured and produced for direct human consumption. It is one of the most cultivated marine fish species in Southern Europe and in the Mediterranean region and its global production exceeded 250 thousand tonnes in 2019 (FAO, 2021). Turkey, Greece, and Egypt account for over 70% of seabream production worldwide, with their share being 39%, 21% and 14%, respectively.

Large-scale production became feasible in the second half of the 1980's when constrains on broodstock maturation and spawning in captivity were overcome (Moretti et al., 1999). Although seabream is predominantly farmed in cages (inshore and offshore), especially in the Mediterranean, production systems also include onshore extensive earthen ponds and lagoons, intensive land-based concrete tanks with a flow-through water supply and RAS. Pre-ongrowing juveniles are usually kept in land-based facilities until individuals reach 2 – 5 g, or 10 – 30 g, in the case of being transferred to open sea conditions for the ongrowing stage (Mozes et al., 2011). In the last decade, production in offshore cages has increased due to limited locations and growing competition with other economic activities for land and coastal use. In addition, issues such as nutrient outputs and escapes to the natural environment, as well as the advantage in controlling abiotic factors, have encouraged the development of inland RAS production.

1.4.2.2. Nutrition of gilthead seabream

Gilthead seabream is a carnivorous marine fish and as such possesses high protein and lipid requirements. Other than fish size and age, protein requirements are dependent upon protein digestibility and amino acid profile. Nevertheless, inclusion of 55% protein in diets for seabream fry (under 5 g), 45% for juveniles of 100 – 200 g, and 40% for 300 – 400 g fish, seemed to be adequate for growth (Lupatsch et al., 2003; Santinha et al., 1996; Vergara et al., 1996b). Currently, commercial feeds contain a fair amount of plant ingredients as protein sources. Feeding fish with diets that incorporate considerable amounts of terrestrial plant ingredients has proven its feasibility, even when feeding carnivorous fish species such as the gilthead seabream, as long as selected crystalline amino acids are added to overturn any deficiency or imbalance in the amino acid profile (Aragão et al., 2020; Gatlin et al., 2007; Hardy, 2010; Oliva-Teles et al., 2015).

The ability to process high levels of dietary lipids depends on the species and is related to the fish feeding habits. Seabream fed diets containing 22% lipid content, using fish oil, showed good growth performance and enhanced protein utilisation when compared with fish fed 15% lipid diets (Vergara et al., 1999). This protein-sparing effect of dietary lipids is discussed in Chapter IV. Marine fish have been fed diets incorporating lipid sources of marine origin, due to their inability to synthesise long-chain polyunsaturated fatty acids (PUFA) (Sargent et al., 2003). Not without its challenges, the substitution of fish oil by single or blends of vegetable oils in diets for gilthead seabream, has proven its viability, at least up to 60 – 70% (Fountoulaki et al., 2009; Izquierdo et al., 2003).

1.5. OBJECTIVES

The sustainability of the aquaculture industry is largely dependent on formulating diets that promote nitrogen retention and maximise fish growth performance. This PhD Thesis is focused on the optimisation of environmentally sustainable and cost-effective feeds for the production of two fish species with great relevance to the World and European aquaculture – Nile tilapia (*Oreochromis niloticus*) and gilthead seabream (*Sparus aurata*). For this purpose, the following goals were proposed:

- Reduce protein inclusion levels in plant-based diets for juvenile Nile tilapia, through adequate amino acid supplementation, to minimise diet environmental impact and increase biological efficiency (**Chapter II**).
- Evaluate how different dietary formulations affect the bioavailability and metabolic fate of ketogenic or glucogenic indispensable amino acids in juvenile gilthead seabream (**Chapter III**).
- Establish how the protein to energy ratios in plant-based diets affect the overall protein metabolism, and particularly of lysine and methionine, in gilthead seabream (**Chapter IV**).
- Assess the influence of different methionine sources (DL-methionine and calcium bis-methionine hydroxyl analogue – MHA-Ca) on the metabolism and growth of Nile tilapia (**Chapter V**).
- Reduce protein and fishmeal contents in diets for gilthead seabream, to promote growth and minimise environmental impacts during unfavourable rearing conditions (**Chapter VI**).

This PhD Thesis intends to provide an integrated overview on the metabolic fate of selected nutrients by combining fundamental and applied studies such as growth performance, diet digestibility and nutrient flux studies.

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Chapter II

Optimising diets to decrease environmental impact of Nile tilapia (*Oreochromis niloticus*) production

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Optimising diets to decrease environmental impact of Nile tilapia (*Oreochromis niloticus*) production

Abstract

Aquaculture is one of the most thriving animal production sectors, and Nile tilapia (*Oreochromis niloticus*) farming represents 8% of total finfish culture. However, the industry sustainability depends on the development of cost-effective and environmental friendly feeds. This study aimed to reduce dietary protein levels in diets for juvenile tilapia and to minimise diet environmental impact while maximising biological efficiency. A growth trial was performed using five isoenergetic plant protein-based diets with decreasing levels of crude protein: 36, 34, 32, 30 and 28% (D36, D34, D32, D30 and D28, respectively). Dietary protein utilisation was assessed by metabolic trials using a radiolabelled amino acid mixture. Tilapia in all treatments showed similar growth performance and feed intake. Feed conversion ratio was significantly higher in fish fed the D28 than the D36 diet, while no differences were found for other treatments. Protein retention was significantly higher in tilapia fed the D30 than the D36 diet. Amino acid catabolism increased in fish fed the D36 diet, though without significant differences in muscle amino acid retention. This study demonstrates that dietary protein levels can be reduced to 30% without hindering tilapia growth and feed conversion ratio, while reducing environmental nitrogen losses.

Keywords: Dietary protein content; Environmental impact; Nile tilapia; Nitrogen retention; Nutrient output.

2.1. INTRODUCTION

The aquaculture sector is currently one of the most prosperous animal production industries. World finfish aquaculture production has been progressively rising, accounting for almost 47% of total fish production (FAO, 2018). On a global scale, tilapias are the second most cultivated finfish group, with Nile tilapia (*Oreochromis niloticus*) accounting for 8% of total finfish produced in 2016 (FAO, 2018). This species is particularly popular due to its fast-growing rates, disease resistance, robustness and ability to adapt to different farming systems (Ng and Romano, 2013).

Aquaculture feed account for 50% of the total production costs with protein being the most expensive nutrient in the diet (Ballester-Moltó et al., 2017). A major challenge for the aquaculture industry is to find new strategies for precision diet formulation that minimise rising feed cost, while implementing more sustainable farming practices that rely on enhanced feeding strategies and high-quality diets. Optimised feeds will not only reduce production costs but will also decrease the impact of fish farming on the environment, namely minimising the release of phosphorus and nitrogen into the environment (Klinger and Naylor, 2012).

Feed cost has been largely determined by dietary protein sources and inclusion levels. Fish meal is considered a reference protein in fish diets, including diets for omnivorous species like tilapia, since it presents a high content of protein with a balanced amino acid profile, it is highly palatable, and it is a source of essential fatty acids, minerals and vitamins (Miles and Chapman, 2006). However, due to the constant rise of fishmeal price and its questionable environmental sustainability, there has been a change in the paradigm of diet formulation (Bureau and Hua, 2010). Several studies focused on fishmeal replacement by more economical and/or environmental friendly sources in diets for Nile tilapia and its hybrids and suggested that the replacement success is dependent on a dietary balanced amino acid profile, among other factors (Figueiredo-Silva et al., 2015; Furuya et al., 2004; Suloma et al., 2014; Trosvik et al., 2012). Nowadays, tilapia feeds include on average 2% of fishmeal (FAO, 2018). The surge of alternative ingredients in diet formulation requires additional attention to the quantitative and qualitative amino acid levels, which determine protein quality.

Response of Nile tilapia to dietary protein levels has been widely studied and, as for other fish species, is dependent on fish size/age, dietary amino acid profile and digestibility (Ng and Romano, 2013). Optimal growth of Nile tilapia fry was obtained with diets containing 45% crude protein (CP), while for fingerlings and advanced juveniles optimal growth performance was observed with diets containing 35% CP (Abdel-Tawwab et al., 2010), and for adults, optimal growth was obtained at 25% CP (El-Saidy and Gaber, 2005). However, the amino acid profile of the experimental diets was not reported in these studies. Tilapia have likely responded to meet the requirements for indispensable amino acids rather than to the dietary protein levels *per se*. Diets with high levels of protein or with an imbalanced amino acid profile may result in increased amino acid catabolism and consequently higher nitrogen losses to the environment (Bureau et al., 2002). Therefore, dietary supplementation with crystalline amino acids provides an opportunity to balance the dietary amino acid profile, while reducing excess levels of dietary protein and thus decreasing environmental impacts due to a reduction of nitrogen excretion (Gan et al., 2012; Gaylord and Barrows, 2009).

Metabolic trials using radiolabelled nutrients allow to obtain an *in vivo* insight of dietary protein utilisation, allowing to determine the proportions of nutrients that are catabolised for energy purposes or retained in the body (Costas, 2011; Rønnestad et al., 2001). This methodology has been previously used, for instance, to demonstrate the importance of balanced amino acid profiles or specific amino acids to increase amino retention (Aragão et al., 2004; Richard et al., 2017) and the impairment of protein retention under specific feeding regimes (Engrola et al., 2010; 2009) or alternative feed ingredients (Rocha et al., 2016a; 2016b).

Currently, the expansion of the aquaculture industry is greatly dependent on the development of sustainable feeds. Given the important production volume of tilapia and the expected growth of aquaculture as a strategy to feed nine billion people in 2050, it is essential to find diets that are cost-effective and environmental friendly. In this context, the aim of this study was to reduce protein inclusion levels in plant protein-based diets for juvenile Nile tilapia, through adequate amino acid supplementation, in order to minimise diet environmental impact while maximising biological efficiency. Furthermore, metabolic trials were performed aiming to obtain an in vivo snapshot of protein utilisation in Nile tilapia juveniles as a function of dietary protein content.

2.2. MATERIALS AND METHODS

2.2.1. Experimental diets

Five isoenergetic diets were formulated differing in their protein levels (36, 34, 32, 30 and 28% crude protein), using plant ingredients as well as meat and bone meal as protein sources (Table 2.1). Diets were formulated to meet the minimum requirements of amino acids, on digestible basis, for Nile tilapia (*Oreochromis niloticus*) juveniles using the AMINOTilapia® software (Evonik Nutrition & Care; Table 2.2). Apparent digestibility coefficients (ADC) of amino acids for the ingredients used were taken from the literature review (Konnert and Masagounder, 2017; Konnert et al., 2017), with ADC values ranging from 86% to 95% for various amino acids in the experimental diets. Diets were supplemented with increasing levels of selected indispensable amino acids and di-calcium phosphate with the decreasing levels of dietary protein to avoid amino acid or mineral imbalances. Upon ingredient grinding with a hammer mill (model SH1, Hosokawa-Alpine, Germany) and its mixing in a double-helix mixer, all diets (pellet size 2.0 mm) were manufactured using a twin-screw extruder (model BC45, Clextral, France) at SPAROS Lda. Upon cold extrusion at 45ºC, diets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, the oil was added to the pellets by vacuum coating (model PG-10VCLAB, Dinnisen, the Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature, in a cool and aerated storage room. Proximate composition and amino acid analysis were determined in all experimental diets, as reported in Tables 2.1 and 2.2, respectively.

2.2.2. Growth trial

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Nile tilapia (Silver Natural Male Tilapia™) juveniles with an average body weight of 2.70 g were obtained from Til-Aqua International B.V. (the Netherlands), and the experiment was conducted at Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licences by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). Upon arrival, fish were acclimatised to the new rearing facilities in a recirculation aquaculture system and were fed a commercial diet (crude protein: 36%; crude fat: 8%).

Table 2.1 Formulation and proximate composition of the experimental diets.

	Diets				
Ingredients (%)	**D36**	**D34**	**D32**	**D30**	**D28**
Soybean meal [a]	48.77	44.39	40.60	39.03	34.12
Corn meal [b]	22.10	26.66	30.83	34.37	39.91
Cottonseed meal [c]	10.00	10.00	10.00	10.00	10.00
Rapeseed meal [d]	8.00	8.00	8.00	8.00	6.52
Meat and bone meal [e]	8.74	7.68	5.99	2.00	1.50
Soybean oil	1.00	1.34	1.79	2.31	2.61
Vitamin & mineral premix [f]	1.00	1.00	1.00	1.00	1.00
Di-calcium phosphate	0.08	0.32	0.80	1.79	2.08
DL-Methionine	0.31	0.36	0.41	0.46	0.52
L-Lysine		0.19	0.43	0.65	0.93
L-Threonine		0.06	0.14	0.21	0.31
L-Tryptophan			0.01	0.03	0.07
L-Isoleucine				0.06	0.17
L-Valine				0.05	0.16
L-Histidine				0.04	0.10
Analysed proximate composition (% as fed basis)					
Dry matter	94.8	94.7	95.4	96.2	96.0
Ash	8.3	7.9	7.7	7.5	6.9
Crude protein	37.9	35.2	33.4	31.3	29.5
Crude fat	4.6	5.0	5.4	5.0	5.4
Total phosphorus	0.9	0.9	0.9	0.9	0.9
Gross energy (MJ kg^{-1})	17.0	16.7	17.1	17.0	17.4

All values are reported as means of duplicate analysis.

[a] Soybean meal: 46.8% crude protein (CP), 2.1% crude fat (CF).

[b] Corn meal: 7.5% CP, 4.2% CF.

[c] Cottonseed meal: 44.2% CP, 3.9% CF.

[d] Rapeseed meal: 33.2% CP, 2.1% CF.

[e] Meat and bone meal: 53.3% CP, 16.1% CF.

[f] Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's.

Table 2.2 Amino acid composition of experimental diets.

			Diets			Evonik recommendation
Analysed values *(mg AA g $^{-1}$ as fed)*	**D36**	**D34**	**D32**	**D30**	**D28**	**AMINOTilapia®** **(digestible basis)**
Methionine	8.2	8.4	8.7	8.8	8.9	7.4
Cysteine	5.3	5.0	4.8	4.7	4.4	
Methionine + Cysteine	13.5	13.4	13.5	13.5	13.3	12.3
Lysine	20.4	19.4	19.5	19.1	19.2	17.6
Threonine	14.0	13.4	13.3	13.1	13.0	11.7
Tryptophan	4.6	4.2	4.1	3.9	4.0	3.7
Arginine	27.6	25.4	23.8	22.1	20.4	16.1
Isoleucine	15.8	14.4	13.5	13.5	13.2	11.2
Leucine	27.6	25.6	24.1	22.8	21.2	15.0
Valine	17.7	16.3	15.3	14.9	14.7	12.4
Histidine	9.3	8.6	8.1	8.0	7.9	7.4
Phenylalanine	18.2	16.7	15.7	14.8	13.7	11.6
Glycine	20.5	18.7	16.9	14.0	12.6	
Serine	17.6	16.3	15.2	14.2	13.1	
Proline	22.1	20.2	18.8	17.2	15.9	
Alanine	18.2	16.8	15.6	14.0	13.1	
Aspartate	37.8	34.7	32.2	30.3	27.5	
Glutamate	64.5	59.5	55.8	52.8	48.7	

All values are reported as means of duplicate analysis.

Juvenile Nile tilapia were reared in 100 L cylindrical tanks in a recirculating aquaculture system. The experimental system was equipped with a mechanical filter, a submerged biological filter and a UV sterilizer. Photoperiod was set at 10 h:14 h (light:dark) cycle, temperature averaged 25.2 ± 0.1°C, and dissolved oxygen (DO) in water was maintained above 80% of saturation (> 6.6 mg L^{-1} DO). Water quality parameters were monitored daily and adjusted when necessary: pH was maintained between 7.7 and 8.2, and the concentration of unionized ammonia and nitrites in water was 0 mg L^{-1} during the whole experimental period. Mortality was monitored daily.

At the end of the trial, each tank was bulk weighed and 10 fish from each tank were individually weighed. Six fish from each tank were euthanised with anaesthetic (1.5 ml L^{-1} of phenoxyethanol), and the whole fish were stored at −20°C until analysis of proximate composition and amino acid content. Fish were deprived of diets for 24 h before initial and final samplings.

2.2.3. Chemical analysis

Raw materials (soybean meal, cornmeal, cottonseed meal, rapeseed meal and, meat and bone meal) were analysed for dry matter, crude protein and amino acid content using NIR (AMINONIR®, Evonik Nutrition & Care) before diet formulation. Before analysis, diets and pooled whole-body fish were finely ground. Chemical analysis followed the standard procedures of the Association of Official Analytical Chemists (AOAC, 2006) and was run in duplicates. Moisture content was determined by drying the samples at 105°C for 24 h and ash content by incineration in a muffle furnace at 500°C for 12 h. Freeze-dried whole-body fish samples and diets were analysed for crude protein (N × 6.25) by wet chemistry (AMINOLab®, Evonik Nutrition & Care) using the combustion/Dumas method; crude fat by petroleum ether extraction using a Soxtherm Multistat/ SX PC (Gerhardt); and gross energy by combustion in an adiabatic bomb calorimeter (Werke C2000, IKA) calibrated with benzoic acid and phosphorous content by digestion at 230°C in a Kjeldatherm block digestion unit followed by digestion at 75°C in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406). Amino acid content in diets and whole-body fish samples was analysed by wet chemistry (AMINOLab®, Evonik Nutrition & Care) using ion-exchange chromatography, except for tryptophan, which was estimated using HPLC.

2.2.4. Calculations

Growth performance parameters and nutrient retention were calculated as follows:

Weight gain (% IBW) = 100 × (FBW − IBW) × IBW^{-1}, where IBW and FBW are the initial and final body weights, respectively.

Daily growth index (DGI) = 100 × (FBW$^{1/3}$ – IBW$^{1/3}$) × days^{-1}.

Feed conversion ratio (FCR) = apparent feed intake × wet weight gain^{-1}, where wet weight gain is: FBW – IBW.

Daily voluntary feed intake (VFI, % IBW day^{-1}) = 100 × crude feed intake × IBW^{-1} × days^{-1}.

Protein efficiency ratio (PER) = wet weight gain × crude protein intake^{-1}.

Nutrient retention (% intake) = 100 × (FBW × final body nutrient content − IBW × initial body nutrient content) / (nutrient intake).

Daily nutrient intake (mg kg^{-1} ABW day^{-1}) = nutrient intake × ABW^{-1} × days^{-1}, where ABW = (IBW + FBW)/ 2.

Daily nutrient gain (mg kg^{-1} ABW day^{-1}) = (final body nutrient content − initial body nutrient content) × ABW^{-1} × days^{-1}.

Daily nutrient loss (mg kg^{-1} ABW day^{-1}) = daily nutrient intake − daily nutrient gain.

2.2.5. Dietary protein utilisation

At the end of the trial, to further understand protein utilisation as a function of dietary protein content, a metabolic trial using a radiolabelled amino acid mixture was performed. Random fish from the higher, intermediate and lower protein dietary treatments (D36, D32 and D28; n = 6 from each treatment) were transferred to the nutrient flux laboratory after being deprived of diets for 24 h. The *in vivo* method of tube-feeding used to perform the metabolic trials was adapted from Costas (2011), which was a modification of the method first described by Rust et al. (1993) and modified by Rønnestad et al. (2001). Briefly, tube-feeding was performed on anaesthetized fish by gently pushing the labelled feed pellets into the oesophagus, using a hollow plastic tube of 1.5 mm inner diameter and a solid piece with a smaller diameter placed inside as a plunger. The diameter and length of the plastic tube was previously tested to avoid injuring the fish oesophagus. An [U-^{14}C]-L-amino acid mixture (3.7 MBq mL^{-1}, American Radiolabeled Chemicals Inc.) was used as a tracer to radiolabel the experimental diets. The tracer was diluted in freshwater Ringer solution, and a known value of the diluted tracer was dispensed on individual pellets of the different experimental diets. The pellets were dried at 50°C for 1 h and reserved for subsequent tube-feeding. The quantity of feed pellets administered to each fish corresponded to 1.3% body weight (one meal). The metabolic fate of the tracer was considered to represent the fate of tracer – dietary protein (Conceição et al., 2007).

Tube-fed fish recovered in clean and aerated freshwater to eliminate residual anaesthetic and monitor for eventual pellet regurgitation. After this period, fish were transferred into individual incubation chambers containing 2 L of freshwater at 25°C. Each chamber was hermetically sealed, supplied with a gentle oxygen flow and connected to a series of three CO_2 traps (each containing 10 ml of 0.5 M KOH). After 24 h of incubation, oxygen flow was stopped and fish were euthanised inside the chambers by a lethal dose of anaesthetic (MS-222 buffered with sodium bicarbonate, Sigma). Fish were removed for sampling (retention fraction), and incubation chambers were resealed for the addition of 1 M HCl in a series of gradual steps, leading to a progressive decrease in water pH causing the diffusion of any remaining $^{14}CO_2$ from the water into the CO_2 traps (catabolism fraction). Samples from CO_2 traps were collected at the end of the acidification process for radioactivity determination. Each fish was weighed, and one fish fillet (muscle) with skin was collected and weighed. Muscle aliquots of 200 mg were totally dissolved in Solvable (PerkinElmer) at 50°C for 24 h. Scintillation cocktail (Ultima Gold XR, PerkinElmer) was added to all samples, and DPM was counted in a TriCarb 2910TR low activity liquid scintillation analyser (PerkinElmer). All counts were corrected for quench and lumex.

Amino acid utilisation was determined as a function of dietary protein content (provided by the feed), based on the metabolic fate of the ^{14}C-amino acid mixture used as a tracer. Relative ^{14}C-amino acid catabolism and relative ^{14}C-amino acid retention in muscle were normalised for fish body weight and expressed as mg of ingested protein per g of fish:

^{14}C-amino acid catabolism = (Ingested protein × $DPM_{Catabolism}$) / (DPM_{Feed} × Fish weight);

^{14}C-amino acid retention = (Ingested protein × DPM_{Muscle}) / (DPM_{Feed} × Fish weight);

where $DPM_{Catabolism}$ was the radioactivity (DPM) found in the sum of all CO_2 traps, DPM_{Muscle} was the radioactivity found in the muscle (fish fillets) and DPM_{Feed} was the radioactivity found in the feed pellets tube-fed to each fish.

2.2.6. Statistical analysis

Data are presented as means ± standard deviation. Data expressed as a percentage were arcsine transformed previously to the statistical analysis (Ennos, 2007). Data were checked for normal distribution and homogeneity of variances. All data were subjected to one-way analysis of variance (ANOVA). Differences among groups were compared by Tukey's multiple comparison test at $P < 0.05$ level of significance. Statistical analyses were performed using the IBM SPSS Statistics 24 software.

2.3. RESULTS

2.3.1. Growth performance and feed utilisation

The overall growth performance of fish fed the experimental diets is presented in Table 2.3. All fish had a fourfold increase in body weight at the end of the experiment, independently of the diet, and no significant differences ($p > 0.05$) were found at the end of the experiment, with mean values ranging from 29.34 to 31.49 g. Fish weight gain or daily growth index (DGI) was not influenced ($p > 0.05$) by the different dietary protein levels.

Feed conversion ratio (FCR) increased with decreasing levels of dietary protein but differed significantly ($p < 0.05$) only between the group fed D28 (1.30 ± 0.05) and those fed the D36 diet (1.16 ± 0.05). Protein efficiency ratio (PER) increased with decreasing levels of dietary protein; therefore, the D28 group presented the highest PER (2.60 ± 0.09) and the D36 presented the lowest (2.27 ± 0.09). The groups fed D28, D30 and D32 diets exhibited no significant differences among them ($p > 0.05$) and were significantly different from the group fed the D36 diet ($p < 0.05$). No differences were detected among treatments concerning the daily voluntary feed intake, which varied from 8.1% to 8.7% IBW/day ($p > 0.05$).

There were no significant differences ($p > 0.05$) in survival among fish fed the experimental diets, which in overall was 98 ± 3%.

Table 2.3 Growth performance and survival of Nile tilapia juveniles fed the experimental diets for 59 days.

	Diets				
	D36	**D34**	**D32**	**D30**	**D28**
Initial body weight, IBW (g)	5.93 ± 1.73	5.93 ± 1.61	5.90 ± 1.75	5.90 ± 1.61	5.89 ± 1.63
Final body weight (g)	31.49 ± 2.19	31.04 ± 0.89	30.93 ± 0.41	30.07 ± 1.66	29.34 ± 1.69
Weight gain (% IBW)	439.82 ± 33.56	413.05 ± 24.95	421.11 ± 3.24	386.35 ± 29.45	385.89 ± 21.66
Daily growth index (DGI)	2.30 ± 0.11	2.26 ± 0.04	2.26 ± 0.03	2.19 ± 0.09	2.16 ± 0.10
Feed conversion ratio (FCR)	1.16 ± 0.05^{b}	1.19 ± 0.06^{ab}	1.18 ± 0.03^{ab}	1.24 ± 0.05^{ab}	1.30 ± 0.05^{a}
Daily voluntary feed intake (% IBW day^{-1})	8.65 ± 0.33	8.30 ± 0.17	8.44 ± 0.27	8.13 ± 0.31	8.48 ± 0.41
Feed intake (g fish^{-1})	29.84 ± 1.27	29.05 ± 0.80	29.38 ± 0.92	28.70 ± 0.90	29.48 ± 1.45
Protein efficiency ratio (PER)	2.27 ± 0.09^{b}	2.37 ± 0.16^{ab}	2.50 ± 0.10^{a}	2.52 ± 0.13^{a}	2.60 ± 0.09^{a}
Survival (%)	100 ± 0	97 ± 5	99 ± 3	96 ± 5	97 ± 3

Values are presented as means ± standard deviation (n = 3, except for IBW where n = 69). Different superscripts within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences ($p > 0.05$).

2.3.2. Whole-body fish composition and nutrient retention

Whole-body fish composition and nutrient retention values are presented in Table 2.4. Fish from the D30 group presented lower moisture content, higher body fat and higher energy values than those fed D34 and D36 diets ($p < 0.05$). Moreover, fish fed the D30 diet showed the highest body protein values although only significantly different ($p < 0.05$) from the D28 and D32 fish. Ash values were highest for the fish from the D30 group but differed significantly only between this group and the D32 and the D36 groups ($p < 0.05$). No significant differences were found among treatments in phosphorous content ($p > 0.05$).

Fish fed the D30 diet exhibited higher body protein retention than those fed the D36 diet (41% vs. 36%, $p < 0.05$). Fish fed the D30 diet presented the highest phosphorous retention when compared with D28 fish ($p < 0.05$). No significant differences were detected among treatments in energy retention ($p > 0.05$).

The retention of the majority of the amino acids followed a similar pattern to that of protein retention, with fish fed the D30 diet presenting a tendency for higher retention values than those fed the D36 diet (Table 2.5). However, significant differences were only found for phenylalanine ($p < 0.05$). The exception to this trend was methionine, which presented the highest retention in fish fed the D36 diet, although no significant differences were found between the D36 and the D30 treatments ($p > 0.05$).

Daily nitrogen gain (Figure 2.1) was similar among treatments (average daily nitrogen gain = 568.55 ± 23.43 mg kg^{-1} ABW day^{-1}, $p > 0.05$), but there were significant differences concerning the values of daily nitrogen losses. Fish fed low-protein diets, D28 and D30, presented the lowest daily nitrogen loss although only significantly different from the D36 group ($p < 0.05$). Fish fed with the D36 diet lose 64% of their daily nitrogen intake, while fish fed with D28 and D30 diets lose 59 – 60% of their daily nitrogen intake. Moreover, fish fed the D30 diet presented higher phosphorous gain and lower phosphorus loss than those fed the D28 diet (Figure 2.2, $p < 0.05$).

Table 2.4 Whole-body composition and nutrient and energy retention of Nile tilapia juveniles fed the experimental diets for 59 days.

	Diets				
Body composition (% wet weight)	**D36**	**D34**	**D32**	**D30**	**D28**
Moisture	76.60 ± 0.01^{a}	75.58 ± 0.14^{bc}	76.31 ± 0.55^{ab}	74.14 ± 0.31^{d}	74.83 ± 0.24^{cd}
Ash	2.36 ± 1.7^{b}	2.57 ± 3.2^{ab}	2.24 ± 0.2^{b}	2.85 ± 0.7^{a}	2.46 ± 1.7^{ab}
Protein	15.52 ± 3.3^{ab}	15.60 ± 1.2^{a}	14.74 ± 4.2^{c}	15.84 ± 0.9^{a}	15.12 ± 2.8^{bc}
Fat	4.67 ± 4.3^{b}	5.11 ± 4.2^{b}	5.44 ± 2.9^{ab}	6.23 ± 2.8^{a}	6.13 ± 1.9^{ab}
Phosphorus	0.45 ± 0.2	0.44 ± 0.4	0.47 ± 0.6	0.55 ± 0.4	0.45 ± 0.3
Energy (MJ kg^{-1})	5.26 ± 0.07^{c}	5.58 ± 0.08^{b}	5.53 ± 0.14^{bc}	6.06 ± 0.11^{a}	5.89 ± 0.09^{a}
Retention (% intake)					
Protein	35.92 ± 1.59^{b}	37.86 ± 2.15^{ab}	37.28 ± 1.77^{ab}	41.14 ± 2.17^{a}	39.97 ± 1.53^{ab}
Phosphorus	40.16 ± 3.43^{ab}	38.59 ± 2.22^{ab}	40.45 ± 7.15^{ab}	47.54 ± 1.65^{a}	35.12 ± 2.52^{b}
Energy	26.62 ± 1.44	28.26 ± 2.34	27.49 ± 1.28	29.20 ± 1.50	26.63 ± 1.13

Values are presented as means ± standard deviation ($n = 3$). Different superscripts within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences ($p > 0.05$).

Table 2.5 Retention of indispensable amino acids (% intake) in Nile tilapia juveniles fed the experimental diets for 59 days.

	Diets				
Amino acids	**D36**	**D34**	**D32**	**D30**	**D28**
Methionine	42.48 ± 1.75^{a}	41.06 ± 1.98^{ab}	36.29 ± 1.50^{bc}	36.96 ± 2.53^{abc}	33.28 ± 1.61^{c}
Cysteine	26.92 ± 0.83	28.31 ± 1.86	27.21 ± 0.50	28.91 ± 1.86	28.00 ± 1.56
Lysine	54.20 ± 2.93	55.71 ± 2.46	51.91 ± 2.49	53.75 ± 3.50	48.72 ± 2.17
Threonine	41.47 ± 1.73	42.12 ± 1.98	39.83 ± 1.59	41.36 ± 2.49	37.92 ± 1.68
Arginine	29.38 ± 1.12	31.23 ± 1.65	30.84 ± 1.45	34.52 ± 2.12	34.50 ± 1.56
Isoleucine	37.46 ± 1.61	39.86 ± 1.86	40.01 ± 1.39	40.78 ± 2.90	37.74 ± 1.86
Leucine	36.52 ± 1.49	38.23 ± 1.71	38.18 ± 1.39	40.94 ± 3.06	40.00 ± 1.82
Valine	36.73 ± 1.77	38.69 ± 1.93	38.84 ± 1.63	40.57 ± 2.53	37.23 ± 1.79
Histidine	37.67 ± 1.76	39.73 ± 2.33	39.91 ± 1.35	41.57 ± 2.39	38.06 ± 2.01
Phenylalanine	30.58 ± 1.17^{ab}	32.48 ± 1.23^{ab}	32.21 ± 1.09^{ab}	34.85 ± 2.17^{a}	34.55 ± 1.71^{a}

Values are presented as means ± standard deviation ($n = 3$). Different superscripts within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences ($p > 0.05$).

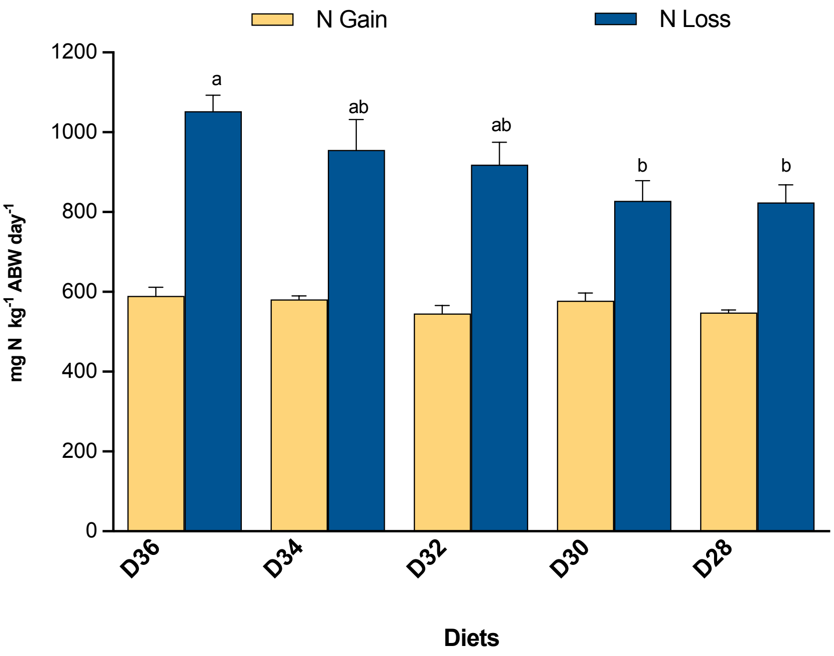

Figure 2.1 Daily nitrogen (N) balance in Nile tilapia juveniles fed the experimental diets for 59 days. Values are presented as means ± standard deviation ($n = 3$). Different letters within the same compartment indicate significant differences ($p < 0.05$) among diets. Absence of letters indicates no significant differences ($p > 0.05$).

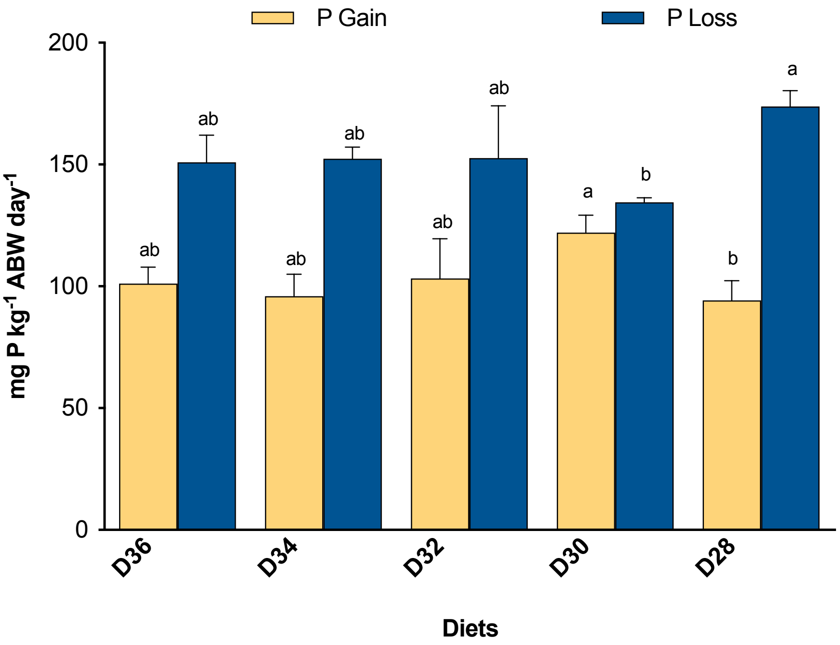

Figure 2.2 Phosphorus (P) balance in Nile tilapia juveniles fed the experimental diets for 59 days. Values are presented as means ± standard deviation ($n = 3$). Different letters within the same compartment indicate significant differences ($p < 0.05$) among diets.

2.3.3. Dietary protein utilisation

The results of the metabolic trials gave an *in vivo* picture on how dietary protein was being utilised. The mean values of amino acid catabolism were lower for the D28 and D34 groups than for the D36 group, although no significant differences were found (Figure 2.3, $p > 0.05$). The highest individual values for amino acid catabolism were also found for fish fed the D36 diet. The dispersion of values for amino acid catabolism was lower in fish fed the D28 diet than in fish fed the D34 and D36 diets.

Regarding relative amino acid retention in muscle, the mean values were quite similar among treatments and no significant differences were found ($p > 0.05$). A higher dispersion of individual values was found for fish fed the D36 diet, and the highest values of amino acid retention in muscle were also found in this treatment.

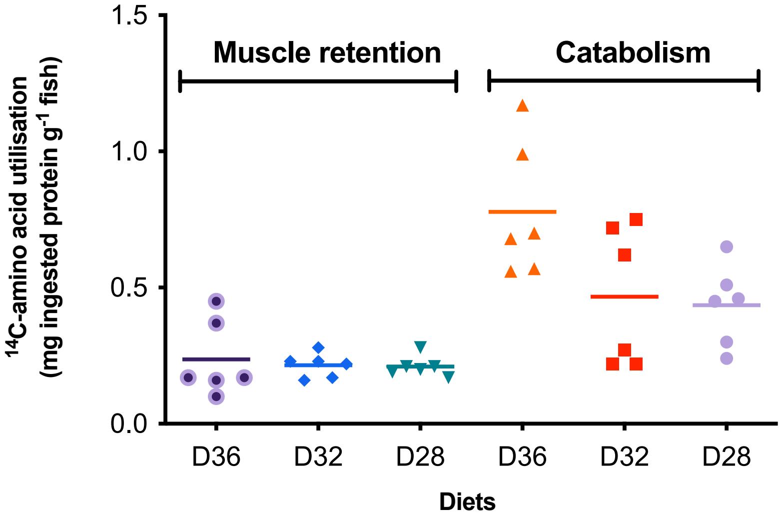

Figure 2.3 Relative ^{14}C-amino acid catabolism and relative ^{14}C-amino acid retention in muscle of Nile tilapia juveniles fed experimental diets with different protein levels (D36, D32, D28). Individual value plots and means (black line) per treatment. Mean values were not significantly different among treatments ($p > 0.05$).

2.4. DISCUSSION

All experimental diets were suitable for the fish; that is, the levels of inclusion of protein in diets of Nile tilapia juveniles had no detrimental effect on survival, weight gain, daily growth index and daily voluntary feed intake. The fish growth performance was similar to other studies (Abdel-Tawwab et al., 2010; El-Saidy and Gaber, 2003; Hernández et al., 2010; Figueiredo-Silva et al., 2015; Silva et al., 2014), particularly taking into account that the experimental diets contained no fish meal nor fish oil in their formulation.

The feed conversion ratio (FCR) increased with decreasing levels of dietary protein, but FCR values only differed significantly between fish fed the lowest (D28) and the highest (D36) protein diets. Feed conversion ratio for Nile tilapia found in the literature varies significantly, which is likely due to discrepancies in experimental diets and conditions, such as feed quality (physical and nutritional), feeding regime, fish age and size. Considering fish size and dietary protein sources (mainly plant ingredients), the FCR values in the present study are lower or within the values found in similar studies (Abdel-Tawwab et al., 2010; El-Saidy and Gaber, 2003; Hernández et al., 2010; Figueiredo-Silva et al., 2015; Silva et al., 2014). Based on the overall growth performance and the FCR observed in the present study, it is implied that the dietary protein levels can be reduced down to 30% without hindering fish growth performance and FCR.

The protein efficiency ratio (PER) was the lowest in fish fed with the highest protein diet (D36) and significantly lower than that of fish fed the lowest protein diets (D28 to D32). These results suggest that fish from the D36 group were less efficient using dietary protein than fish fed the lower protein diets (D28, D30 or D32). A decrease in PER with increasing levels of dietary protein has been previously found in studies with tilapia (Abdel-Tawwab et al., 2010; Kpundeh et al., 2015) and other freshwater fish species (Chen et al., 2010).

Another indication that fish fed the D36 diet did not use dietary protein efficiently is that this group exhibited the lowest protein retention. The highest protein retention and whole-body protein content were found for fish fed the D30 diet. In genetically improved farmed tilapia (GIFT), the same pattern of higher protein retention in fish fed low-protein diets has been observed, although no significant differences were found on whole-body protein content (Kpundeh et al., 2015). The whole-body lipid content increased concomitantly with decreasing dietary protein content. In the lower protein diets, more cornmeal and soybean oil were added to balance the dietary energy level. Energetic efficiencies of digestible fat and carbohydrate were shown to be much higher than that of protein for Nile tilapia (Schrama et al., 2018). This suggests that differences in energy utilisation of macronutrients should be considered while balancing the diets for dietary energy.

The retention of several indispensable amino acids (Cys, Arg, Ile, Leu, Val, His, Phe) followed a similar trend to that of protein, with fish fed the D30 diet showing the highest retention. Interestingly, methionine retention tended to decline in the reduced protein diets with increasing levels of supplementation. While no significant difference was found in the retention between fish fed the D30 and the D36 diet, methionine retention was significantly lower in fish fed the D28 diet than in those fed the D34 or D36 diets. Amino acid profile was quite similar among all diets, including the methionine content. This indicates that tilapia did not effectively utilise the methionine (Met) supplement in the D28 diet for protein synthesis. As the dietary protein level decreased from 36 to 28%, cysteine level declined from 5.3 to 4.4 mg g^{-1} diet, which resulted in Met + Cys (13.3 – 13.5 mg g^{-1}diet) being more limiting than methionine (8.2 – 8.9 mg g^{-1} diet) alone. Therefore, methionine in the lower protein diets was likely converted to cysteine to meet other metabolic needs of fish, explaining the reduced methionine retention. Previous studies have shown the importance of an adequate amino acid supplementation to tilapia diets with low inclusion levels of fishmeal to balance the dietary amino acid profile (Figueiredo-Silva et al., 2015; Furuya et al., 2004; Trosvik et al., 2012). In the present study, the level of supplementation seems to be adequate in the D30 diet.

Fish fed the D28 diet presented the lowest phosphorous retention, expressed as a percentage of intake, and the highest daily phosphorus losses. This indicates that the dietary phosphorous supplement (in the form of di-calcium phosphate) was not effectively used by the tilapia when incorporated at the highest level. Previous works showed that the digestibility of the different sources of inorganic phosphate is different and may be quite low (Morales et al., 2018). The group fed the D30 diet presented the highest phosphorous retention, along with the highest protein retention, indicating that this diet promotes higher nutrient retention and consequently lower environmental impacts due to a reduction in nutrient outputs.

In the current experiment, nitrogen losses increased with the increasing dietary protein content. Similarly, Abdel-Tawwab et al. (2010) found increased levels of unionized ammonia in rearing water with increasing dietary protein content. Higher nitrogen losses are due to lower protein digestibility and/or higher protein catabolism. As the metabolic trials showed, a significant part of the nitrogen losses is due to a higher protein catabolism. Amino acid catabolism in fish results in ammonia production and subsequent excretion to the surrounding environment (Ip et al., 2001). Therefore, the tendency for an increased amino acid catabolism found in fish fed the D36 diet results probably in higher ammonia excretion, and hence in higher nitrogen output to the environment.

In the metabolic trials, fish from all groups were tube-fed one meal. Consequently, the dietary intake of all fish was similar during the metabolic trial, although the amount of ingested protein was different since these diets contained different protein levels. Therefore, fish fed high-protein diets ingested more protein than the ones fed the lower protein diets. This reproduces what happened during the growth trial, since the voluntary feed intake was similar among treatments. Therefore, although fish fed the D36 diet ingested more protein than the other fish groups, they retained the same protein in the muscle as fish from the D28 and D32 groups. This is in line with the results from the growth trial, as no significant differences were found in growth performance among treatments. Growth is essentially protein deposition (Carter and Houlihan, 2001), and the metabolic trials showed that fish fed the high-protein diets were inefficient in retaining protein in the target organ – the muscle. This corroborates the results found for PER and protein retention, since fish fed high-protein diets did not retain more protein.

The results from the metabolic trials indicate that the D36 diet had excessive protein content, since in these fish the protein utilisation to energy purposes increased while protein retention in muscle was maintained at the same level as in fish fed the low-protein diets. The average protein catabolism was not significantly different among treatments, perhaps due to the high dispersion of values, indicating that different individuals presented different metabolic responses within a short period of time (24 h of incubation). Nevertheless, a tendency for higher catabolism in fish fed D36 diet was observed. If one translates this snapshot of 24 h from the in vivo trial for 59 days of the feeding trial, this could explain why fish fed D360 diet that were eating the same amount of feed, but with higher protein intake, did not perform better. Previously, it was suggested that dietary excess protein that was not utilised efficiently might have been used for energy by tilapia (Abdel-Tawwab et al., 2010). The present work reinforces that suggestion and indicates that this excessive dietary protein content ultimately results on the use of amino acids as energy source and consequently on higher environmental impacts, due to increased nitrogen outputs.

In conclusion, the present study demonstrates that it is possible to reduce protein levels in juvenile Nile tilapia diets to 30% without compromising fish growth and FCR, while reducing nitrogen and phosphorus losses to the environment. Additionally, this diet promotes protein retention. Therefore, reducing the dietary protein levels to 30% with an appropriate amino acid supplementation seems an advisable strategy to guarantee an economical and environmental sustainability of tilapia production.

2.5. ACKNOWLEDGEMENTS

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Chapter III

Metabolic fate is defined by amino acid nature in gilthead seabream (*Sparus aurata*) fed different diet formulations

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Metabolic fate is defined by amino acid nature in gilthead seabream (*Sparus aurata*) fed different diet formulations

Abstract

The sustainability of the Aquaculture industry relies on the optimisation of diets to promote nitrogen retention and maximise fish growth. Since optimal growth is conditioned by amino acids balance, and factors, like species and size, may affect amino acids absorption efficiency, the aim of this study was to assess how different dietary formulations affect the bioavailability and metabolic fate of distinct amino acids in gilthead seabream juveniles. Amino acids (lysine, tryptophan and methionine) were selected based on their ketogenic and/or glucogenic nature. Gilthead seabream juveniles were fed practical diets with different protein and lipid contents: 44P21L, 44P18L, 40P21L and 40P18L. After three weeks of feeding, fish were tube-fed the correspondent diet labelled with ^{14}C-lysine, ^{14}C-tryptophan or ^{14}C-methionine. Amino acid utilisation was determined based on the proportion of ^{14}C-amino acid evacuated, retained in gut, liver and muscle, or catabolised. This study revealed that the metabolic fate of the amino acids was mainly determined by their nature. Tryptophan was significantly more evacuated than lysine or methionine, independently of the diet, indicating a lower availability for metabolic purposes. Amino acid retention in gut and liver was equally affected by dietary treatments and amino acid nature. In these tissues, fish fed the 40P18L diet presented a higher retention than fish fed the high protein diets (44P21L and 44P18L), with lysine being one of the most retained amino acids. Methionine was more retained in the muscle independently of the diet, indicating a higher availability of this amino acid for growth and other metabolic purposes. Lysine was preferentially catabolised, independently of the diet and of any dietary amino acid imbalance, indicating that catabolism in seabream juveniles is predominantly ketogenic. The present study underpins the importance of the bioavailability and metabolic fate of amino acids to formulate cost-effective fish diets that ultimately maximise fish growth.

Keywords: Amino acids; Ketogenic; Glucogenic; Catabolism; Bioavailability; Diet formulation.

3.1. INTRODUCTION

The sustainability of the aquaculture sector is largely dependent on optimised diets that promote nitrogen retention and maximise fish growth performance. Growth is essentially protein deposition and optimised growth requires the knowledge on the ideal dietary amino acid profile (Carter and Houlihan, 2001). This profile may vary with fish species and developmental stage (Conceição et al., 1998; 2003a). Optimal growth is conditioned by the efficiency of absorption of each amino acid and precise information on the rates of absorption and catabolism, *i.e.* the relative bioavailability of individual amino acids (Conceição et al., 2003b). The absorption of individual amino acids in the gut relies on a multitude of transport systems, some of which reveal a broad substrate sensitivity: several amino acids may be uptaken by the same transporter and also one amino acid may be transported by more than one system. Therefore, absorption of amino acids is influenced by several factors such as their concentration in the intestinal lumen, transport affinity and capacity for each transporter, and the amount of each transporter present in the epithelium (Conceição et al., 2011; Vilella et al., 1990). To maximise fish growth, amino acids are required to be available in tissues at an optimum ratio, as imbalances will lead to amino acid losses for energy purposes rather than for protein synthesis (Halver and Hardy, 2003).

Amino acids may be catabolised in multiple pathways and be classified into three different categories according to their metabolic fate: ketogenic, glucogenic, and ketogenic plus glucogenic. Ketogenic amino acids, such as lysine, are catabolised only to produce acetyl-CoA, a precursor of ketone bodies or long-chain fatty acids. Lysine is mainly involved in protein synthesis. However, this indispensable amino acid also plays a role in the structure and function of collagen, through its metabolite hydroxylysine, and, along with methionine, is required for the synthesis of carnitine. Glucogenic amino acids (*e.g.* methionine) can generate metabolic intermediate molecules such as alpha-keto acids, pyruvate and oxaloacetate that are crucial to many processes that occur in animals, and be converted into glucose through gluconeogenesis (Wu, 2013). Methionine is a sulphur-containing amino acid involved in protein synthesis, transmethylation, remethylation and transsulfuration reactions. Additionally, ketogenic plus glucogenic amino acids, such as tryptophan, can be catabolised into both acetyl-CoA and 4-5 carbon unit metabolites. Tryptophan is the least abundant amino acid in fish proteins. As a precursor of the neurotransmitter serotonin and of the hormone melatonin, it regulates stress and immune, as well as behavioural responses in fish (Hoglund et al., 2019; Hoseini et al., 2019).

Currently, fish feeds contain a fair amount of vegetable ingredients as protein sources. Feeding fish with diets that incorporate considerable amounts of terrestrial plant ingredients has proven its feasibility, even when feeding carnivorous fish species such as the gilthead seabream (*Sparus aurata*), as long as selected crystalline amino acids are added to overturn any deficiency or imbalance in the amino acid profile (Gatlin et al., 2007; Hardy, 2010; Oliva-Teles et al., 2015). If imbalances occur, amino acid catabolism and consequent metabolic losses are inevitable and excessive nitrogen may be lost to the aquatic environment (Bureau et al., 2002). It is important to understand how fish discriminate among the different amino acids when fed different diet formulations to ensure an optimised utilisation under any diet, rearing conditions, and developmental stage. The gathered knowledge will help formulate balanced diets to allow fish to reach their full growth potential.

The aim of the present study was to assess how different dietary formulations could affect the bioavailability and metabolic fate of selected indispensable amino acids like lysine, tryptophan and methionine. Indispensable amino acids were selected based on their ketogenic, glucogenic or ketogenic and glucogenic nature. Gilthead seabream juveniles were fed practical diets incorporating different protein and/or lipid levels. Metabolic flux assays were performed by tube-feeding ^{14}C-labelled diets to estimate evacuation, retention and catabolism of amino acids.

3.2. MATERIALS AND METHODS

3.2.1. Experimental diets

Four experimental diets were formulated with two levels of crude protein (44 and 40% CP) and two levels of crude lipids (21 and 18% CL) using practical ingredients. Diets were designated 44P21L, 44P18L, 40P21L and 40P18L according to their protein and lipid contents (Table 3.1). High protein diets, 44P21L and 44P18L, included 27% fishmeal and incorporated 54% and 51% plant ingredients as protein sources, respectively. The lower protein diets (40P21L and 40P18L) contained 21% fishmeal and 57 – 60% plant protein sources. Fish oil to rapeseed oil ratio was kept at approximately 1.5 to 1.0 in all diets. Selected crystalline indispensable amino acids and mono-calcium phosphate were supplemented in diets, whenever necessary, to avoid any indispensable amino acids or phosphorus imbalance. Formulation, proximate composition and amino acid analysis of diets are presented in Tables 3.1 and 3.2.

Table 3.1 Formulation and proximate composition of experimental diets.

Ingredients (%)	**44P21L**	**44P18L**	**40P21L**	**40P18L**
Fishmeal SP [a]	22.00	22.00	16.00	16.00
Fishmeal [b]	5.00	5.00	5.00	5.00
Soy protein concentrate [c]	6.40	6.00	6.90	6.40
Wheat gluten [d]	7.00	6.40	6.00	6.00
Corn gluten [e]	11.00	11.00	10.00	10.00
Soybean meal [f]	12.00	12.00	12.00	12.00
Rapeseed meal [g]	5.00	5.00	5.00	5.00
Sunflower meal [h]	4.00	5.00	5.00	5.00
Wheat meal [i]	2.90	4.90	7.00	10.50
Whole peas [j]	3.00	4.00	5.00	5.00
Fish oil [k]	10.60	8.52	10.85	9.00
Rapeseed oil [l]	6.60	5.68	6.85	5.70
Vitamin and Mineral Premix [m]	1.00	1.00	1.00	1.00
Vitamin E [n]	0.10	0.10	0.10	0.10
Choline chloride [o]	0.10	0.10	0.10	0.10
Betaine HCl [p]	0.50	0.50	0.50	0.50
Soy lecithin [q]	0.50	0.50	0.50	0.50
Guar gum [r]	0.50	0.50	0.50	0.50
Antioxidant powder [s]	0.20	0.20	0.20	0.20
Mono-calcium phosphate [t]	1.10	1.10	1.40	1.40
L-Lysine [u]	0.30	0.30	0.10	0.10
L-Threonine [v]	0.20	0.20		
Proximate composition (% as fed)				
Dry matter	94.17	93.73	94.69	94.05
Ash	8.53	9.22	8.10	8.20
Crude protein	44.72	43.88	40.40	40.43
Crude lipids	20.32	17.65	21.38	18.40
Total phosphorus	1.15	1.08	1.20	1.19
Gross energy (MJ kg^{-1})	22.33	21.71	22.11	21.66

All values are reported as means of duplicate analysis.

[a] Super-Prime: 68% crude protein (CP), 8% crude fat (CF), Pesquera Diamante, Peru.
[b] CONRESA 60: 65% CP, 10% CF, Conserveros Reunidos S.A., Spain.
[c] Soycomil P: 63% CP, 8% CF; ADM, The Netherlands.
[d] VITAL: 80% CP, 7.5% CF; Roquette Frères, France.
[e] Corn gluten meal: 61% CP, 6% CF; COPAM, Portugal.
[f] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF; CARGILL, Spain.
[g] Defatted rapeseed meal: 34% CP, 2% CF; Premix Lda., Portugal.
[h] Solvent extracted dehulled sunflower meal: 43% CP, 3% CF, MAZZOLENI SPA, Italy.
[i] Wheat meal: 10% CP, 1.2% CF; Casa Lanchinha, Portugal.
[j] Yellow peas: 19.6% CP, 2.2% CF; Ribeiro e Sousa Lda., Portugal.
[k] Sopropêche, France.
[l] J.C. Coimbra Lda., Portugal.
[m] PREMIX Lda., Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocoferol acetate 100 mg; sodium menadione bisulphate 25 mg; retinyl acetate 20,000 IU; DL-cholecalciferol 2,000 IU; thiamin 30 mg; riboflavin 30 mg; pyridoxine 20 mg; cyanocobalamine 0.1 mg; nicotinic acid 200 mg; folic acid 15 mg; ascorbic acid 1,000 mg; inositol 500 mg; biotin 3 mg; calcium panthotenate 100 mg; choline chloride 1,000 mg; and betaine 500 mg. Minerals (g or mg/kg diet): cobalt carbonate 0.65 mg; copper sulphate 9 mg; ferric sulphate 6 mg; potassium iodide 0.5 mg; manganese oxide 9.6 mg; sodium selenite 0.01 mg; zinc sulphate 7.5 mg; sodium chloride 400 mg; calcium carbonate 1.86 g; and excipient wheat middlings.
[n] ROVIMIX E50, DSM Nutritional Products, Switzerland.
[o] ORFFA, The Netherlands.
[p] Beta-Key 95%, ORFFA, The Netherlands.
[q] Lecico P700IPM, LECICO GmbH, Germany.
[r] Guar gum, Seah International, France.
[s] Paramega PX, KEMIN EUROPE NV, Belgium.
[t] MCP: 22% P, 18% Ca; Fosfitalia, Italy.
[u] L-Lysine HCl 99%, Ajinomoto Eurolysine SAS, France.
[v] L-Threonine: 98%; EVONIK Nutrition & Care GmbH, Germany.

All experimental diets were manufactured by SPAROS Lda. (Olhão, Portugal). Diets (pellet size 2 mm) were produced by extrusion by means of a pilot-scale twin-screw extruder (CLEXTRAL BC45; Clextral, France) with a screw diameter of 55.5 mm and temperature ranging from 105°C to 110°C. Upon extrusion, all batches of extruded feeds were dried in a vibrating fluid bed dryer (model DR100; TGC Extrusion, France). Following drying, pellets were allowed to cool at room temperature and subsequently the oil fraction was added under vacuum coating in a Pegasus vacuum mixer (PG-10VCLAB; DINNISEN, The Netherlands). Throughout the duration of the trial, experimental diets were stored at room temperature, in a cool and aerated storage room.

3.2.2. Fish husbandry

Experiments were carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Animal protocols were performed under Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal.

Gilthead seabream (*Sparus aurata*) juveniles (9.1 ± 2.9 g) were kept at the Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). Throughout the experimental feeding period, fish were maintained in 40 L cylinder-conical tanks in a recirculation aquaculture system (water temperature: 20.1 ± 1.2°C; salinity: 32.2 ± 1.4 psu; initial fish density: 4.8 kg m^{-3}). Fish were assigned one of the four experimental diets and fed by automatic feeders, twice a day at a ratio of 3% body weight day^{-1} for three weeks.

Table 3.2 Amino acid composition of experimental diets.

Amino acids *(mg AA g^{-1} as fed)*	**44P21L**	**44P18L**	**40P21L**	**40P18L**
Arginine	45.1	42.9	31.4	29.8
Histidine	12.8	12.1	9.8	10.0
Lysine	43.0	40.8	23.8	22.4
Threonine	21.7	19.6	15.3	16.3
Isoleucine	24.1	23.3	16.9	18.0
Leucine	41.4	39.6	32.9	33.8
Valine	24.2	23.2	20.2	20.6
Methionine	13.0	12.2	10.0	10.1
Phenylalanine	27.7	26.7	20.2	19.9
Cystine	3.1	3.0	2.5	2.3
Tyrosine	23.1	21.9	15.9	14.5
Aspartic acid + Asparagine	54.6	51.5	33.1	31.3
Glutamic acid + Glutamine	104.5	99.4	65.4	64.3
Alanine	26.3	24.9	21.0	20.6
Glycine	29.0	27.6	24.1	24.2
Proline	32.2	30.4	26.1	26.2
Serine	26.4	25.1	19.7	20.1

All values are reported as mean of duplicate analysis.

3.2.3. Metabolic flux assays

After three weeks of feeding the experimental diets, fish from each dietary treatment (44P21L, 44P18L, 40P21L and 40P18L) were transferred to the nutrient flux laboratory after being fasted for 24 h. To determine the metabolic fate of the selected indispensable amino acids as a function of their nature (ketogenic and/or glucogenic) and of the dietary treatment, fish were tube-fed with the correspondent experimental diets labelled with one of the following tracers: ^{14}C-lysine (Perkin Elmer, USA), ^{14}C-tryptophan or ^{14}C-methionine (American Radiolabeled Chemicals Inc., USA). Six fish per diet and tracer were subjected to this procedure (6 fish x 4 diets x 3 tracers, n = 72 fish in total).

3.2.3.1. Diet labelling with ^{14}C-amino acids

Radiolabelled ^{14}C-lysine, ^{14}C-tryptophan or ^{14}C-methionine were diluted in Ringer solution for marine fish and a known amount of the tracer was dispensed with a micropipette on individual pellets ("hot" pellets) of the correspondent experimental diet (44P21L, 44P18L, 40P21L and 40P18L). Additionally, another set of pellets were labelled with a non-radiolabelled solution of blue food colourant ("cold" pellets), used to monitor possible pellet regurgitation. After the labelling, all pellets were dried at 50°C for 1 h. Two "hot" and one "cold" pellet per fish and diet were loaded into a hollow plastic tube of 1.5 mm inner diameter and stored for subsequent tube-feeding. The number of pellets corresponded to 0.5% of fish body weight. Prior to tube-feeding, the amount of radioactivity (disintegrations per minute; DPM) of ten individual "hot" pellets from each experimental diet labelled with each tracer was determined in a TriCarb 2910TR low activity liquid scintillation analyser (Perkin Elmer) after adding the scintillation cocktail (Ultima Gold XR, Perkin Elmer).

3.2.3.2. Tube-feeding trial

The *in vivo* methodology of tube-feeding was adapted from Costas (2011), which was adapted for juvenile fish from the procedure described by Rønnestad et al. (2001) and was previously published by Teodósio et al. (2021) (Chapter V). Anaesthetised fish (50 mg L^{-1} 2-phenoxyethanol, Sigma-Aldrich, Spain) were taken out of the seawater with a fish net and transferred onto a dry plastic tray. The previously loaded plastic tube containing "hot" and "cold" pellets was inserted into the fish mouth and the feed pellets were gently pushed directly into the oesophagus using a smaller diameter solid piece as a plunger. The diameter and length of the plastic tube was previously tested to avoid injuring the fish oesophagus. This procedure lasted approximately 10 sec. The metabolic fate of the tracer (^{14}C-lysine, ^{14}C-tryptophan or ^{14}C-methionine) was considered to represent the fate of the tracee in the diets (Conceição et al., 2007).

Tube-fed fish were placed into a tank with clean and aerated seawater to eliminate any residual anaesthetic in skin and gills and monitored for eventual pellet regurgitation ("cold" pellet). After, the fish were transferred into individual incubation chambers containing 2 L of seawater at 20 ºC. Chambers were hermitically sealed and supplied with a gentle oxygen flow. Each individual chamber was connected to a series of CO_2 traps (n = 3), each containing 10 mL of 0.5 M KOH, so that $^{14}CO_2$ produced by the fish from catabolism of ^{14}C-amino acids was collected. After an incubation period of 18 h, oxygen flow was stopped, fish were euthanised inside the metabolic chamber by a lethal dose of anaesthetic (1000 mg L^{-1} of MS-222 buffered with sodium bicarbonate) and removed for tissue sampling. After fish were removed, the chambers were resealed, and the incubation seawater was acidified gradually with 0.1 M HCl. This led to a decrease in pH causing any remaining $^{14}CO_2$ in the seawater to be trapped in the CO_2 traps.

3.2.3.3. Metabolic budget determination

Incubation seawater samples were collected from each chamber to determine the amount of radioactivity (DPM) present in the water (n = 5). Individual samples from each CO_2 trap were also taken for radioactivity determination. The amount of radioactivity present in the seawater resulted from evacuated (non-absorbed) ^{14}C-amino acid, while the one present in the $^{14}CO_2$ traps resulted from ^{14}C-amino acid catabolism.

From each fish, the gastrointestinal tract (gut, from the oesophagus to the hindgut), liver and skin-on fillets (muscle) were sampled to assess ^{14}C-amino acid retention in the different body fractions. Fish digestive tract was previously washed in Ringer solution for marine fish to ensure that no alimentary bolus was present before DPM determination. All fish tissues were fully dissolved by adding an appropriate volume of Solvable™ (Perkin Elmer) at 50ºC for 24 h. To all samples from incubation seawater, $^{14}CO_2$ traps, gut, liver, and muscle fractions, Ultima Gold XR scintillation cocktail (Perkin Elmer) was added and DPM counted in a TriCarb 2910TR low activity liquid scintillation analyser. All samples were corrected for quench and lumex.

Amino acid utilisation in seabream juveniles as a function of the amino acids' nature and diet formulation was determined based on the percentage of ^{14}C-amino acid evacuated, retained in the different tissues or catabolised, as follows:

$$Evacuation\ (\%) = (DPM_{SW}/DPM_{Total}) \times 100$$
$$Gut\ Retention\ (\%) = (DPM_{Gut}/DPM_{Total}) \times 100$$
$$Liver\ Retention\ (\%) = (DPM_{Liver}/DPM_{Total}) \times 100$$
$$Muscle\ Retention\ (\%) = (DPM_{Muscle}/DPM_{Total}) \times 100$$
$$Catabolism\ (\%) = (DPM_{Traps}/DPM_{Total}) \times 100$$

Where DPM_{Total} is the sum of the radioactivity (DPM) found in the incubation seawater (DPM_{SW}), gut (DPM_{Gut}), liver (DPM_{Liver}), muscle (DPM_{Muscle}) and CO_2 traps (DPM_{Traps}) fractions.

3.2.4. Chemical analysis

Chemical analysis followed standard procedures of the Association of Official Analytical Chemists (AOAC, 2006) and were run in duplicates. Before analysis, diets were finely ground. Dry matter was determined by drying the samples at 105°C for 24 h and ash content by incineration in a muffle furnace at 550°C for 6 h. Freeze-dried diets were analysed for crude protein (N x 6.25) using a Leco nitrogen analyser (Model FP-528; Leco Corporation, St. Joseph, USA); crude fat by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany); gross energy by combustion in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany) calibrated with benzoic acid; phosphorus content by digestion at 230°C in a Kjeldatherm block digestion unit followed by digestion at 75°C in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406). Diets total amino acid profile was determined by ultra-high-performance liquid chromatography (UPLC) on a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Samples were pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using AccQ Tag method (Waters, USA) after acid hydrolysis (HCl 6 M at 116°C for 24 h in nitrogen-flushed glass vials). Tryptophan content was not determined since it is partially destroyed by acid hydrolysis. Amino acids were identified by retention times of standard mixtures (Waters) and pure standards (Sigma-Aldrich). Instrument control, data acquisition and processing were achieved by the use of Waters Empower software.

3.2.5. Data analysis

Data from the metabolic flux assays are presented as mean ± standard deviation. Data expressed as a percentage were arcsine square root transformed previously to the statistical analysis (Ennos, 2012). All data were checked for normal distribution and homogeneity of variances. Differences among amino acids metabolic fate as a function of their ketogenic and/or glucogenic nature, and of the diet were identified by two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test at $P < 0.05$ level of significance. Statistical analyses were performed using the IBM SPSS Statistics 26 software.

Additionally, metabolic flux assays data were subjected to a principal component analysis (PCA) to verify differences between diet formulations and amino acids' nature and find potential clusters of observations. PCA was carried out using the standard prcomp R function in the auto-scaled matrices. Score plots were generated for the two first principal components (PC1 and PC2) using the ggbiplot package for R. Loadings for PC1 and PC2 were calculated to determine the weight of each original variable in the corresponding PCs. All analysis were carried out using the open-source software R version 4.0.4.

To assess any dietary amino acid imbalances of the experimental diets, dietary A/E ratios (Arai, 1981) were calculated, on a weight basis, as [each indispensable amino acid (IAA) content x (total IAA content)$^{-1}$ x 1000], and plotted against previously published A/E ratios for gilthead seabream juveniles (Kaushik, 1998). Cysteine and tyrosine were included with the IAA, since they can only be synthesised from methionine and phenylalanine, respectively. A deficiency or excess for a given IAA calculated as [$(A/E_{Diet} – A/E_{Fish})$ x A/E_{Fish}^{-1}] x 100, was assumed to occur and to be considered potentially limiting in the diet, when the dietary A/E ratio was at least 10% lower or above than that of the fish.

3.3. RESULTS

In all dietary treatments, the proportion of the tube-fed ^{14}C-tryptophan evacuated was significantly higher compared to ^{14}C-lysine or ^{14}C-methionine (Figure 3.1). The evacuated ^{14}C-tryptophan recovered in the incubation seawater varied from 57% to 67% for fish fed the 40P18L and 44P21L diets, respectively. The proportion of ^{14}C-lysine evacuated ranged from 21% in fish fed the 44P21L diet to 33% in 44P18L fed fish. Concerning ^{14}C-methionine evacuation, the proportion that was not absorbed varied from 27% to 42% in fish fed the 44P18L and 44P21L diets, respectively. No significant differences were detected between lysine and methionine evacuation in fish fed all the experimental diets. The assessment of ^{14}C-amino acids utilisation in gilthead seabream juveniles fed experimental diets with distinctive dietary protein and/or lipid content showed that the dietary treatments (44P21L, 44P18L, 40P21L and 40P18L) had no influence on the evacuation of lysine, tryptophan and methionine, but rather strongly dependent on their ketogenic and/or glucogenic nature.

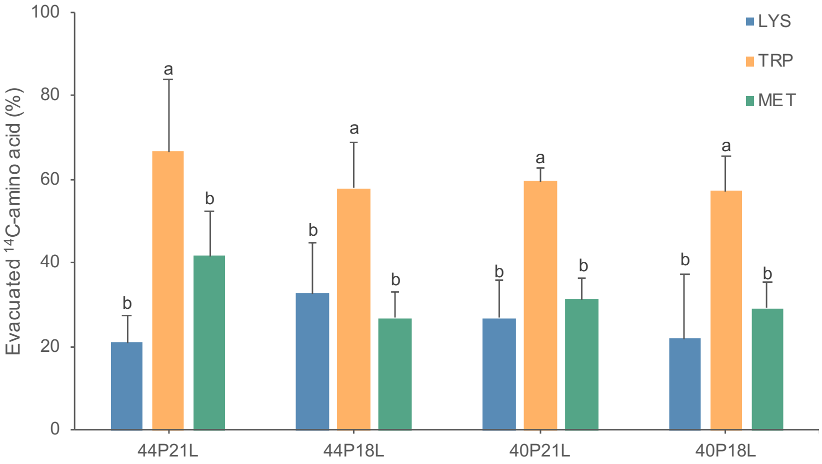

Figure 3.1 Proportion (%) of the total tube-fed ^{14}C-amino acids lysine (LYS), tryptophan (TRP) and methionine (MET) that was evacuated in gilthead seabream juveniles fed 44P21L, 44P18L, 40P21L or 40P18L diets. Values are presented as means ± standard deviation ($n = 6$ fish for each diet and tracer). Letters (a, b) represent significant differences among amino acids' metabolic fate as a function of their nature ($p < 0.05$).

Amino acids retention in the gut (Figure 3.2A) was affected by the diet formulations, although post-hoc tests did not determine which treatments were significantly different. In general, fish fed the 40P21L and the 40P18L diets presented higher retention values for all amino acids than fish fed the high protein diets. Additionally, the nature of the amino acid influenced their retention in this tissue. Lysine was significantly more retained than methionine in all dietary treatments. Tryptophan retention in the gut was similar to lysine or methionine.

Retention of the selected amino acids in the liver was influenced equally by the amino acid nature and by the diet formulation (Figure 3.2B). Fish fed a higher amount of dietary protein (44P21L and 44P18L) presented lower amino acid retention in the liver, but only significantly different when compared to the diet with less protein and lipids (40P18L). Concerning the nature of the amino acids, lysine was more retained in the liver than tryptophan, while methionine presented intermediate levels.

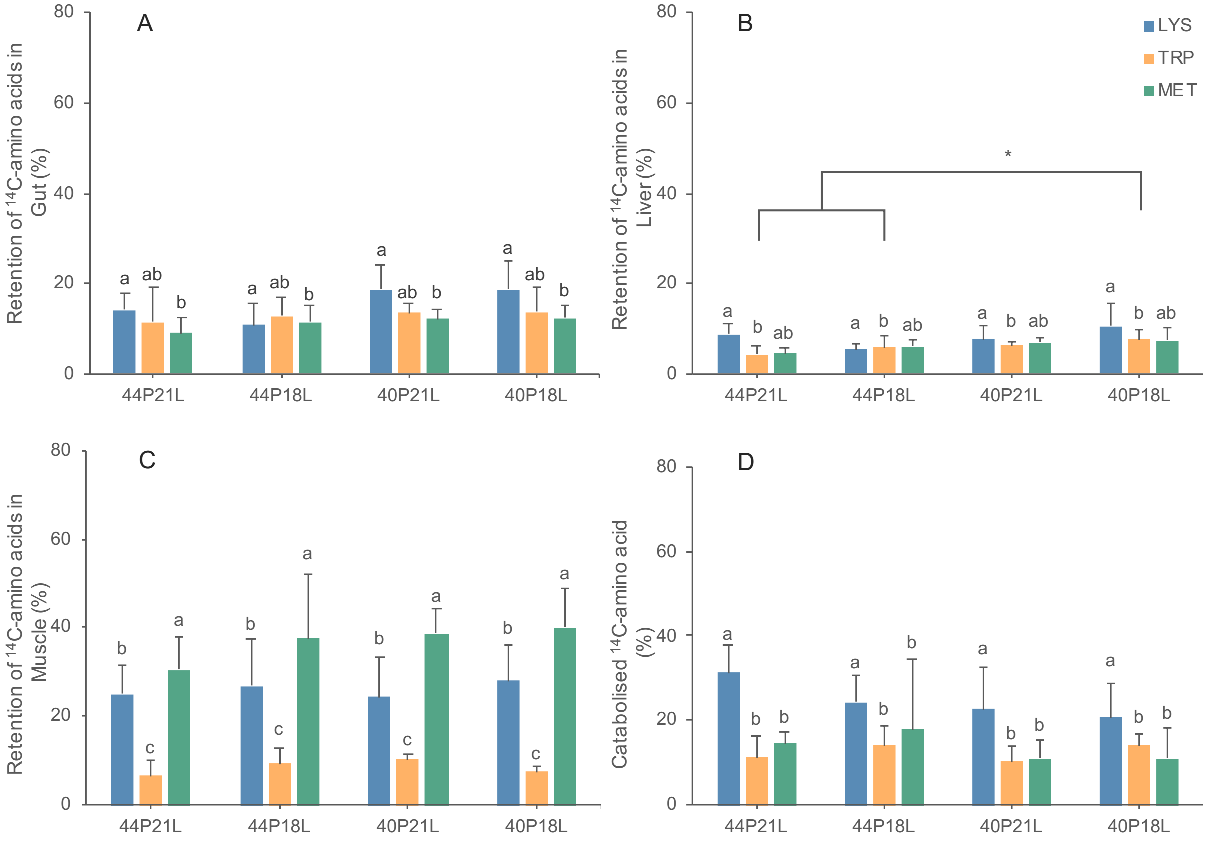

Figure 3.2 Proportion (%) of the total tube-fed ^{14}C-amino acids lysine (LYS), tryptophan (TRP) and methionine (MET) that was retained in the gut **(A)**, liver **(B)** and muscle **(C)**, or catabolised **(D)** in gilthead seabream juveniles fed 44P21L, 44P18L, 40P21L or 40P18L diets. Values are presented as mean ± standard deviation (*n* = 6 fish for each diet and tracer). Letters a, b, c represent significant differences among amino acids metabolic fate as a function of their nature, and * denote significant differences among dietary treatments ($p < 0.05$).

Methionine was preferentially retained in the muscle (Figure 3.2C). Although no significant differences were found among fish fed the different diets, the proportion of ^{14}C-methionine retained in the muscle ranged from 30% in fish fed the 44P21L to 40% in 40P18L fed fish. Moreover, the retention of methionine in the muscle was significantly higher than lysine and tryptophan that presented retention values of 26% and 8%, respectively, for all dietary treatments.

The amino acid's nature significantly influenced their catabolism, independently of the diet. Lysine was significantly more catabolised than tryptophan and methionine (Figure 3.2D). Catabolised lysine varied from approximately 21% in fish fed the 40P18L diet to 31% in

44P21L fed fish. The percentage of tube-fed ^{14}C-tryptophan and ^{14}C-methionine that was catabolised was around 12% and 14%, respectively, for all dietary treatments.

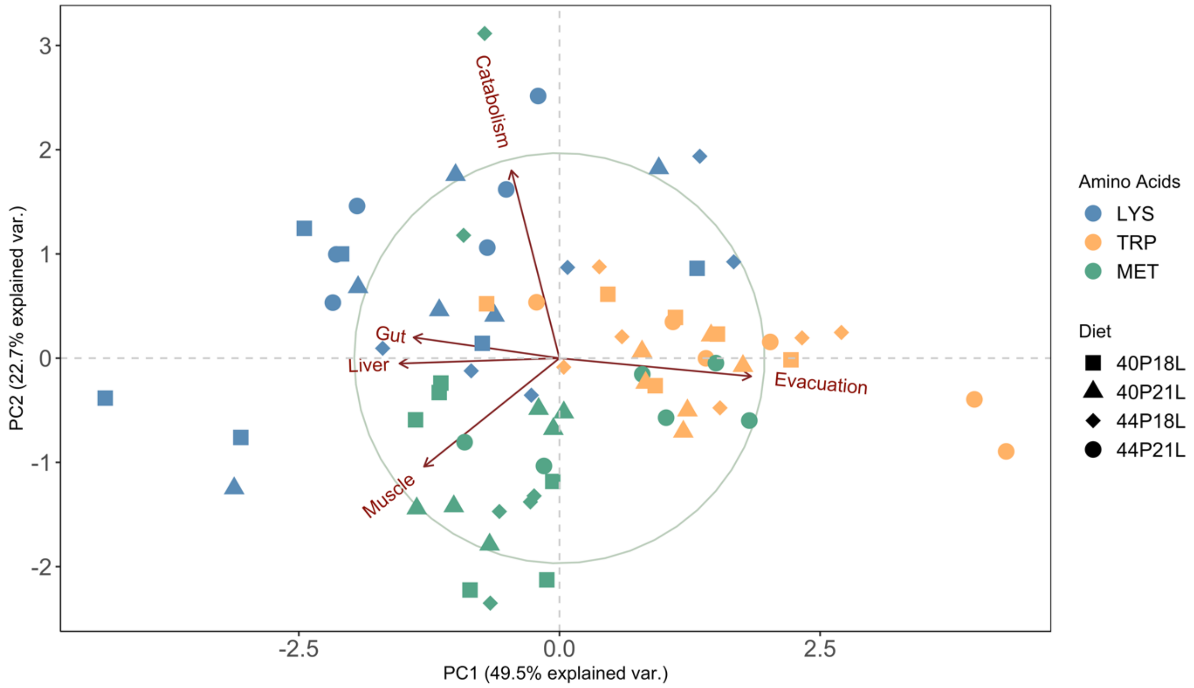

Figure 3.3 Principal component analysis (PCA) of the metabolic flux assays data generated by gilthead seabream juveniles fed different diets (44P21L, 44P18L, 40P21L and 40P18L) labelled with ^{14}C-amino acids lysine (LYS), tryptophan (TRP) and methionine (MET). Each point represents the projection of an individual sample in the PC1 and PC2 axis. Each dietary treatment is identified by a unique shape and each ^{14}C-amino acid as a unique colour, as indicated in the legend.

Principal component analysis (PCA) was used to reduce the complexity of the data from the metabolic flux assays. PCA confirmed that the nature of the amino acids, not the diet formulations, was responsible for the differences observed (Figure 3.3). PC1 and PC2 accounted for 49.5% and 22.7% of the total variability of the data. The analysis of the score plots indicated that tryptophan data was separated from lysine and methionine along the PC1 axis, and that lysine and methionine were separated from each other along the PC2 axis. Evacuation and retention in the liver were the loadings that contributed the most for the dissimilarities observed between fish fed ^{14}C-tryptophan labelled diets and fish fed diets labelled either with ^{14}C-lysine or ^{14}C-methionine. On the other hand, catabolism and muscle retention were the loadings responsible for the differences between fish fed ^{14}C-lysine labelled diets and fish fed diets labelled with ^{14}C-methionine.

Although diets were supplemented with selected crystalline indispensable amino acids, comparison of the dietary A/E ratios with the A/E ratios of fish revealed that all diets presented some amino acid imbalances (Figure 3.4). Lysine was balanced in the high protein diets 44P21L and 44P18L (Figure 3.4A and B), however, lower protein diets 40P21L and 40P18L (Figure 3.4C and D), presented a deficiency of 20% and 24%, respectively. Phenylalanine and tyrosine were found to be in excess in all diets (102% to 112%), while methionine and cysteine were deficient (~20% in the case of the higher protein diets and 14% for diets with lower protein content).

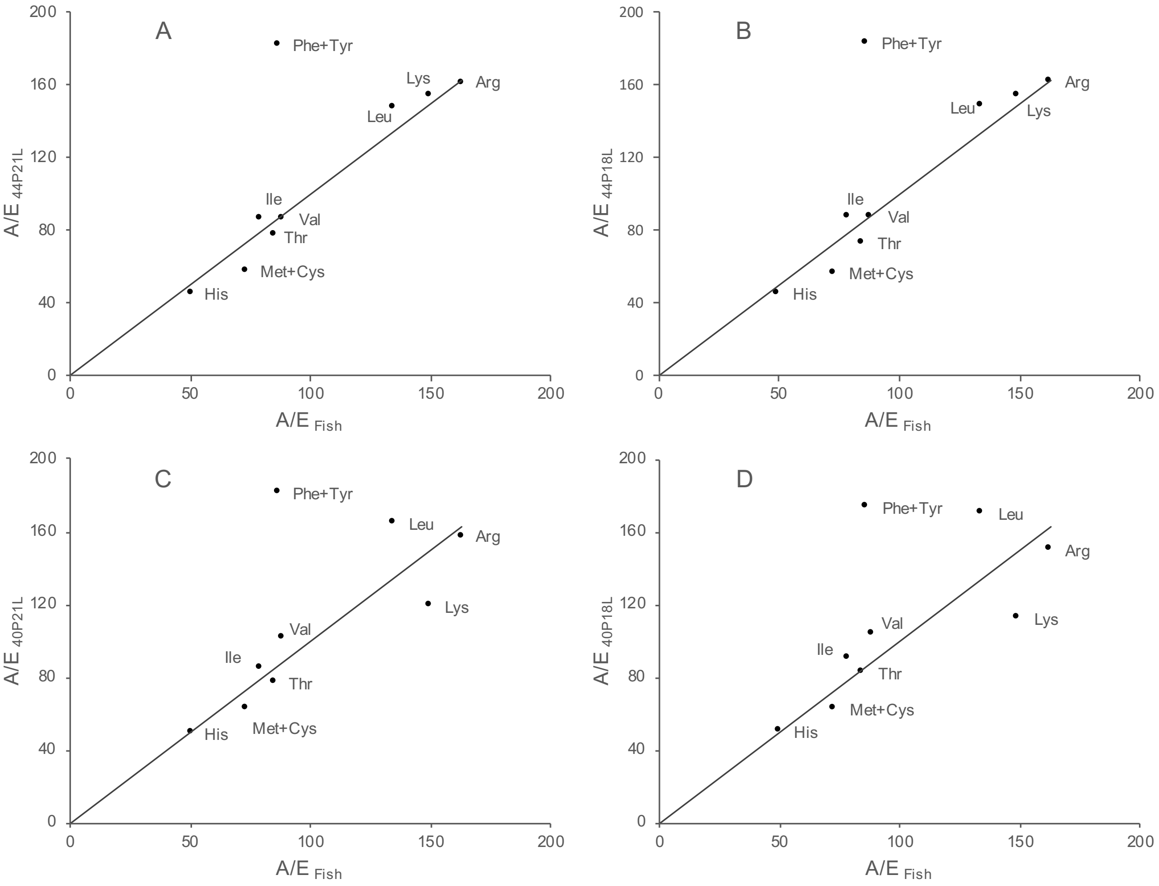

Figure 3.4 Comparison of the A/E ratios of the experimental diets **(A)** 44P21L, **(B)** 44P18L, **(C)** 40P21L, and **(D)** 40P18L with the A/E ratios of gilthead seabream juveniles.

3.4. DISCUSSION

Metabolic flux assays for ^{14}C-lysine, ^{14}C-tryptophan or ^{14}C-methionine were used to assess the bioavailability and metabolic fate of the selected indispensable amino acids as a function of their nature (ketogenic and/or glucogenic) and of the different dietary formulations in gilthead seabream juveniles. The metabolic budget of the selected amino acids was mainly affected by their nature rather than the diet formulation.

Principal component analysis (PCA) confirmed that data were clustered by amino acid and not by dietary treatment. Additionally, evacuation was the variable that contributed the most for the formation of two groups along the PC1 axis, one clustering observations from fish fed all diets labelled with ^{14}C-tryptophan and another that clustered data from fish fed ^{14}C-lysine and ^{14}C-methionine labelled diets. In the current study, tryptophan evacuation was substantially higher than the evacuation of lysine and methionine (60% *versus* 26% and 32%, respectively). Similarly, the metabolic budget determination in Senegalese sole (*Solea senegalensis*) juveniles and in white seabream (*Diplodus sargus*) larvae, also revealed that a higher proportion of ^{14}C-tryptophan was evacuated (41% and 30%, respectively) when compared to all the other indispensable amino acids (Costas, 2011) or to methionine and arginine (Saavedra et al., 2008a). Data on tryptophan digestibility is limited, yet juvenile rainbow trout (*Oncorhynchus mykiss*) showed lower digestibility of tryptophan compared to all other indispensable amino acids independently of the dietary treatment (Dabrowski and Dabrowska, 1981). Free amino acids are transported across the brush-border membrane through carrier-mediated transport systems (Rønnestad et al., 2007; Sire and Vernier, 1992). The same transporter may be responsible for the uptake of several amino acids and consequently some amino acids may interfere with the intestinal uptake of others (Bakke-McKellep et al., 2000; Vilella et al., 1990). TAT1 (Slc16a10) is responsible for the transport of the aromatic amino acids (phenylalanine, tyrosine and tryptophan) in animal cells (Broer and Palacin, 2011; Hyde et al., 2003). The calculation of the A/E ratios showed that in the present study all the experimental diets presented a surplus of phenylalanine and tyrosine. Therefore, it is reasonable to assume that a dietary excess of the two amino acids may interfere with the uptake of tryptophan since they compete for the same transporter. The high levels of phenylalanine and tyrosine present in all experimental diets, may have potentially increased the evacuation of tryptophan resulting in its low availability for metabolic purposes. The current findings highlight the importance of feeding fish with balanced amino acids diets since not only deficiencies but also surplus may have implications on amino acid absorption and utilisation.

The proportion of amino acids retained in the gut and liver of seabream juveniles was affected not only by their nature but also by the dietary treatments. Fish fed the 40P18L diet retained more amino acids in the liver fraction than fish fed the high protein diets (44P21L and 44P18L). These results suggest that when fish are fed lower protein diets, amino acids may be temporarily retained in liver to ensure its availability for metabolic purposes. Furthermore, 18 h after tube-feeding, lysine, which is considered an indicator of protein synthesis, was one of the most retained amino acids in the gut and liver of seabream juveniles. The relatively high retention in these tissues may be associated with the constant cellular turnover occurring in the former and with the crucial role of the latter in energy homeostasis. In general, tryptophan retention in the tissues was low: 13%, 6% and 8% was found in the gut, liver and muscle of juvenile seabream, respectively. It is worth mentioning that the majority of the retained tryptophan (± 48%) was found in the gut, as also observed in Senegalese sole juveniles (Costas, 2011). This may be due to tryptophan being the precursor of serotonin, which is mainly synthesised in the gastrointestinal tract (Gershon, 2004).

The present study showed that most of the absorbed methionine was retained in the muscle. This is in agreement with the results obtained with tracer studies in Senegalese sole juveniles and larvae, as well as white seabream larvae (Costas, 2011; Pinto et al., 2013; Rønnestad et al., 2000; Saavedra et al., 2008a). Moreover, the current work revealed that the retention of methionine in the muscle was significantly higher than lysine and tryptophan retention. PCA results corroborate these findings, demonstrating that fish fed all diets labelled with ^{14}C-methionine were clustered together according to data from muscle retention. Dietary methionine supplementation was shown to improve growth performance in several fish species [Belghit et al., 2014; Figueiredo-Silva et al., 2015; Teodósio et al., 2021(Chapter V)] and its involvement in muscle growth and in the regulation of the expression of genes related to myogenesis has been established (Alami-Durante et al., 2018). Additionally, as a precursor of S-adenosylmethionine (SAM) and of polyamines, methionine is involved in DNA and protein methylation as well as in cell proliferation and differentiation (Li et al., 2009; Wu, 2009). The fact that methionine was preferentially retained in the muscle of fish fed all experimental diets, suggests the importance of this amino acid to be available in this tissue for growth and other metabolic purposes.

Inevitably, a fraction of the absorbed amino acids is catabolised and used for energy rather than growth (Halver and Hardy, 2003). This is especially true if imbalances in the amino acid profile occur. Amino acid catabolism occurs mainly in the liver where amino acids are either converted into others or directed to the tricarboxylic acid (TCA) cycle. Once they enter the TCA cycle, amino acids can either be oxidised to generate energy, channelled towards fatty acid synthesis or gluconeogenesis (Wu, 2013). Although gluconeogenesis occurs in the liver, in fish this pathway is thought to be less significant for amino acids compared to their oxidation (Ballantyne, 2001; van Waarde, 1988). Previous metabolic studies in gilthead seabream (Rocha et al., 2016a; 2016b) and Senegalese sole (Navarro-Guillén et al., 2017) using a ^{14}C-amino acid mixture demonstrated that only a small percentage of absorbed amino acids were converted into lipids or other metabolites, where glucose was included. The current findings revealed that 25% of the tube-fed ^{14}C-lysine was detected in ^{14}C-CO$_2$ traps, significantly more than methionine or tryptophan. Similarly, metabolic budget determination in Senegalese sole juveniles revealed that lysine was significantly more catabolised than all the other indispensable amino acids (Costas, 2011). According to the A/E ratio analysis, all experimental diets exhibited a deficit in sulphur amino acids (methionine + cysteine). Besides, the higher protein diets, 44P21L and 44P18L, met gilthead seabream requirements for lysine, although the low protein diets, 40P21L and 40P18L, were deficient in this amino acid. Nevertheless, lysine, and not methionine, was significantly more catabolised than the other amino acids, independently of the diet. PCA results confirmed that data from the catabolism of fish fed all diets labelled with ^{14}C-methionine and ^{14}C-lysine grouped these amino acids in two different clusters along the PC2 axis. Fish have the ability to regulate their amino acid metabolism with various enzymes being responsible for regulating the differential use of individual amino acids (Ballantyne, 2001). In fact, several studies have demonstrated that selective amino acid retention and catabolism occurs (Aragão et al., 2004; Saavedra et al., 2008a; 2008b). The present results revealed that gilthead seabream juveniles discriminate between the use of different amino acids and that catabolism seems to be predominantly ketogenic.

3.5. CONCLUSION

The assessment of ^{14}C-amino acid utilisation in gilthead seabream juveniles fed different diet formulations showed that amino acids' bioavailability and metabolic fate were mainly determined by their nature. Tryptophan evacuation may be related to its affinity to intestinal transporters and interaction among different amino acids. The low availability of tryptophan should be considered when optimising fish diets formulations. Methionine was found to be preferentially retained in the muscle, most likely to be used for growth. Catabolism was mainly ketogenic, independently of the diet and of any dietary amino acid imbalance. The *in vivo* approach is a valuable tool that allows a fine-tunning of diet formulation. Optimisation of diets considering the amino acids bioavailability will maximise protein retention in fish and is a viable solution to develop cost-effective fish diets.

3.6. ACKNOWLEDGEMENTS

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Chapter IV

Amino acid metabolism in gilthead seabream is differently affected by the dietary protein to energy ratios

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Amino acid metabolism in gilthead seabream is differently affected by the dietary protein to energy ratios

Abstract

The optimisation of the dietary protein to energy ratio (P/E) has proven to influence protein utilisation and/or growth performance in several fish species. This study intended to unravel the bioavailability and metabolic fate of ketogenic (lysine) and glucogenic (methionine) amino acids in gilthead seabream juveniles fed vegetable diets with different P/E ratios. Seabream juveniles were fed two isonitrogenous diets (45% crude protein) differing in crude lipid content (20 and 14%): LowP/E (P/E = 20.0 mg protein kJ^{-1}) and HighP/E (P/E = 21.4 mg protein kJ^{-1}). After three weeks of feeding, fish were tube-fed the respective diet labelled with ^{14}C-protein (L-amino acid mixture), ^{14}C-lysine or ^{14}C-methionine. Protein, lysine and methionine utilisation was determined based on the proportion of ^{14}C-amino acid evacuated, retained in liver and muscle as free or protein-bound amino acid, or catabolised. This study revealed that a decrease in the P/E ratio by increasing the dietary lipid content resulted in lower amino acid evacuation. Furthermore, methionine absorption was less efficient than most of the other protein-bound amino acids. Amino acid retention in the liver was unaffected by the dietary P/E ratio. In addition, the results indicate that amino acids are being retained as protein in this tissue and not only temporarily available in the free pool. The amount of free amino acids retained in the muscle of fish fed the LowP/E diet was significantly higher than in the HighP/E fed fish due to a higher retention of lysine and methionine, but not ^{14}C-protein. Therefore, a decrease in the dietary P/E ratios affect amino acid utilisation and may favour the retention of indispensable over dispensable amino acids in the muscle free pool. Additionally, in fish fed both diets, methionine catabolism was significantly lower than lysine or protein, reinforcing that this amino acid is preferentially spared for metabolic functions and not used as an energy source. In contrast, increasing the dietary P/E ratio decreased lysine catabolism and increased its availability for growth or other metabolic purposes. The bioavailability and metabolism of individual dietary amino acids should be considered when optimising P/E ratios in diets for gilthead seabream juveniles to improve diet utilisation and fish performance.

Keywords: Protein/energy ratio; Protein-sparing effect; Protein utilisation; Amino acid utilisation; Methionine; Lysine.

4.1. INTRODUCTION

The aquaculture industry has responded to global environmental awareness. Although fish growth performance is still one of the main concerns of the sector, maximisation of growth is pursued without disregarding the reduction of nutrient outputs to the aquatic environment and by the inclusion of more sustainable feed sources. Therefore, optimisation of diets to enhance growth while promoting nitrogen retention is key to higher sustainability standards.

Protein utilisation is influenced by numerous factors, including digestibility of ingredients, dietary protein and lipid content, amino acid profile and protein to energy ratio (P/E) (Halver and Hardy, 2003). The optimisation of the P/E ratios has proven to influence protein utilisation and/or growth performance in several fish species. For instance, a decrease in dietary P/E ratios by increasing lipid, and consequently energy content, has been shown to have a protein-sparing effect in salmonids (Cho and Bureau, 2001). In addition, it has led to improved growth performance and protein efficiency in species such as gilthead seabream, *Sparus aurata* (Santinha et al., 1999; Vergara et al., 1996), European seabass, *Dicenthrarchus labrax* (Dias et al., 1998), common dentex, *Dentex dentex* (Skalli et al., 2004), Nile tilapia, *Oreochromis niloticus* (Fernandes et al., 2016) and yellow croaker, *Larimichthys polyactis* (Ma et al., 2020). Japanese seabass (*Lateolabrax japonicus*) fed diets with three levels of protein, at three distinct lipid levels, showed that at each protein level an increase in dietary lipids resulted in improved growth, although the protein-sparing effect of lipids was more pronounced in fish fed the diet with the lowest protein content (Ai et al., 2004). In addition to improved growth, an increase in dietary lipid content was shown to enhance the activity of digestive enzymes and reduce the proportion of dietary protein catabolised by blunt snout bream *Megalobrama amblycephala* (Li et al., 2010; 2012). In contrast, in white seabream (*Diplodus sargus*) fed diets varying in protein (15% and 28%) and lipids (12% and 16%), growth performance was unaffected by the dietary P/E ratios but rather by the dietary protein content (Ozório et al., 2006). Moreover, Senegalese sole (*Solea senegalensis*) growth performance improved with increasing dietary P/E ratio, independently of the protein level, suggesting that this species has a low tolerance to high dietary lipid content (Borges et al., 2009; 2012). Consequently, the optimisation of diet formulations need to consider not only dietary P/E ratios but also the specificities of the species.

It is essential to consider the bioavailability and metabolism of individual dietary amino acids when pursuing the maximisation of fish growth through protein utilisation. Amino acids must be available in animal tissues at an optimum ratio since imbalances will increase amino acid catabolism (Halver and Hardy, 2003), leading to metabolic losses and augmenting nitrogen outputs to the environment (Bureau et al., 2002). In a study performed in seabream juveniles fed plant-based diets incorporating different protein and/or lipid levels, the bioavailability and metabolic fate of selected amino acids was determined as a function of their ketogenic or glucogenic nature and dietary formulations (Chapter III). This study revealed that the metabolic fate of lysine and methionine was mainly influenced by their ketogenic or glucogenic nature rather than the diet formulation. While a significant proportion of the ingested lysine (ketogenic amino acid) was catabolised, methionine (glucogenic amino acid) was preferentially retained in the muscle. Lysine and methionine are considered the two most limiting amino acids in plant-based diets and their dietary supplementation has been shown to improve growth performance in several fish species [Belghit et al., 2014; Richter et al., 2021; Takagi et al., 2001; Teodósio et al., 2021a (Chapter V)]. Lysine, an indicator of protein synthesis, is involved in the formation of collagen, improving fish muscle integrity and fillet texture (Liu et al., 2017; Oliveira et al., 2017) and in lipid metabolism, although the precise mechanism is still poorly understood (Huang et al., 2021). In addition to protein synthesis, methionine is involved in a multitude of physiological and metabolic functions such as the regulation of the expression of genes related to myogenesis (Alami-Durante et al., 2018), DNA and protein methylation as well as cell proliferation and differentiation (Li et al., 2009; Wu 2009).

The current work intends to unravel the bioavailability and metabolic fate of ketogenic (lysine) and glucogenic (methionine) amino acids in gilthead seabream juveniles fed vegetable diets with different P/E ratios. Metabolic flux assays using ^{14}C-labelled diets were used to estimate evacuation, retention, and catabolism of protein (amino acid mixture), lysine and methionine.

4.2. MATERIAL AND METHODS

4.2.1. Diets

Two isonitrogenous diets (45% crude protein) were formulated differing in crude lipid content (20 and 14%), using fishmeal and vegetable meals as protein sources (Table 4.1). Diets were designated LowP/E and HighP/E according to their crude protein to gross energy (P/E) ratio: diet LowP/E had a gross energy content of 22.3 kJ g^{-1} and presented a P/E ratio of 20.0 mg protein kJ^{-1}; while diet HighP/E contained 21.0 kJ g^{-1} of energy and had a P/E ratio of 21.4 mg protein kJ^{-1}. Diets were supplemented with selected crystalline indispensable amino acids and mono-calcium phosphate to avoid any indispensable amino acids or phosphorus imbalance.

Formulation, proximate composition, and amino acid analysis of diets are presented in Tables 4.1 and 4.2.

Upon grinding with a hammer (model SH1, Hosokawa-Alpine, Germany) and its mixing in a double-helix mixer, all diets (pellet size 2 mm) were manufactured using a pilot-scale twin-screw extruder (CLEXTRAL BC45; Clextral, France) at SPAROS Lda. (Olhão, Portugal). Upon extrusion at 105°C to 110°C, diets were dried in a vibrating fluid bed dryer (model DR100; TGC Extrusion, France). Following drying, pellets were allowed to cool at room temperature and subsequently the oil fraction was added under vacuum coating in a Pegasus vacuum mixer (PG-10VCLAB; DINNISEN, The Netherlands). Throughout the duration of the trial, experimental diets were stored at room temperature, in a cool and aerated storage room.

4.2.2. Fish husbandry

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Animal protocols were performed under Group-C licenses by the Direção Geral de Alimentação e Veterinária, Portugal.

Gilthead seabream (*Sparus aurata*) juveniles (11.6 ± 4.3 g) were kept at the Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). Throughout the experimental feeding period, fish were maintained in 40 L cylinder-conical tanks in a recirculating aquaculture system (water temperature: 20.1 ± 1.2°C; salinity: 32.2 ± 1.4 psu; initial fish density: 4.8 kg m^{-3}). Fish were assigned one of the two experimental diets and fed by automatic feeders, twice a day at 3% body weight day^{-1} for three weeks.

4.2.3. Metabolic flux assays

After three weeks of feeding the experimental diets, fish from each dietary treatment were transferred to the nutrient flux laboratory (CCMAR, Portugal) after 24 h of fasting. To determine the effect of P/E ratios on the overall metabolism of amino acids, fish were tube-fed with the respective diet (LowP/E or HighP/E) labelled with ^{14}C-protein ([U-^{14}C]-protein hydrolysate (L-amino acid mixture); American Radiolabeled Chemicals Inc., USA). The effect of the P/E ratios on the metabolism of lysine and methionine was assessed by tube-feeding fish with the correspondent diet labelled with ^{14}C-Lys ([U-^{14}C]-L-lysine; Perkin Elmer, USA) or ^{14}C-Met ([1-^{14}C]-L-methionine; American Radiolabeled Chemicals Inc.). Six fish per diet and tracer were subjected to this procedure (6 fish x 2 diets x 3 tracers, *n* = 36 fish in total).

4.2.3.1. Diet labelling with ^{14}C-tracers

Radiolabelled ^{14}C-protein, ^{14}C-Lys or ^{14}C-Met were diluted in Ringer solution for marine fish and a known value of the tracer was dispensed with a micropipette on individual pellets ("hot" pellets) of the experimental diets (LowP/E and HighP/E). A non-radiolabelled solution of blue food colourant was dispensed in another set of pellets ("cold" pellets) used to monitor possible pellet regurgitation. All pellets were dried at 50°C for 1 h. Two "hot" and one "cold" pellet per fish and diet were loaded into a hollow plastic tube of 1.5 mm inner diameter and stored for subsequent tube-feeding. The number of pellets per fish corresponded to 0.5% of fish body weight. Prior to tube-feeding, the amount of radioactivity (disintegrations per minute; DPM) of ten individual pellets from each experimental diet labelled with each tracer was determined in a TriCarb 2910TR low activity liquid scintillation analyser (Perkin Elmer) after adding the scintillation cocktail (Ultima Gold XR, Perkin Elmer).

4.2.3.2. Tube-feeding trial

The *in vivo* methodology of tube-feeding (Rønnestad et al., 2001a) was adapted for juvenile fish by Costas (2011) and was previously published by Teodósio et al. (2021a - Chapter V). Anaesthetised fish (50 µL L^{-1} 2-phenoxyethanol, Sigma-Aldrich, Spain) were transferred onto a dry plastic tray. Fish were tube-fed the "hot" and "cold" pellets, in approximately 10 sec, taking the necessary measures to guarantee that the fish oesophagus was not injured. The metabolic fate of the tracer (^{14}C-protein, ^{14}C-Lys or ^{14}C-Met) was considered to represent the fate of the tracee in the diets (Conceição et al., 2007).

After tube-feeding, fish were placed in clean and aerated seawater to eliminate any residual anaesthetic in skin and gills and monitored for eventual pellet regurgitation. Once recovered, fish were transferred into individual incubation chambers containing 2 L of seawater at 20 ºC. Chambers were hermitically sealed and provided with a gentle oxygen flow. Each individual chamber was connected to a series of CO_2 traps, each containing 10 mL of 0.5 M KOH. After an incubation period of 18 h, the oxygen flow was stopped, and fish were euthanised inside the chamber by a lethal dose of anaesthetic (1000 mg L^{-1} of MS-222 buffered with sodium bicarbonate). After the removal of the fish for tissue sampling, chambers were resealed. Acidification of the incubation seawater was done gradually by adding 0.1 M HCl, leading to a decrease in pH and to the diffusion of any remaining $^{14}CO_2$ in the water to the CO_2 traps.

4.2.3.3. Metabolic budget determination

Seawater samples were collected from each individual chamber to determine the amount of radioactivity present in the incubation water resulting from evacuated (non-absorbed) ^{14}C-amino acids. To assess ^{14}C-amino acid retention, liver and skin-on fillets (muscle) from each fish were sampled. Amino acid retention in the liver and muscle of fish was evaluated in the free pool and in protein-bound fractions (from hereafter designated as *Free* and *Bound*, respectively). To analyse the radioactivity present in the *Free* fractions, all tissues were incubated at 4°C for 24 h with 6% (w/v) trichloroacetic acid (TCA) with periodical stirrings. After this period, tissues were transferred to a clean vial and the TCA samples were analysed for radioactivity (DPM). Subsequently, all tissues were fully dissolved by adding an appropriate volume of Solvable™ (Perkin Elmer) at 50 ºC for 24 h. Liver was analysed as whole whereas two samples of muscle were used for DPM counting. Additionally, individual samples from each CO_2 trap, representing ^{14}C-amino acid catabolism, were taken for radioactivity determination. Scintillation cocktail (Ultima Gold XR; Perkin Elmer) was added to all samples for radioactivity determination in a TriCarb 2910TR low activity liquid scintillation analyser. All samples were corrected for quench and lumex.

Amino acid metabolic fate in gilthead seabream juveniles as a function of the dietary P/E ratio was determined based on the percentage of ^{14}C-tracer evacuated, retained in *Free* or *Bound* fractions, or catabolised, as follows:

$$Evacuation\ (\%) = \ (DPM_{SW}/DPM_{Total}) \times 100$$
$$Retention\ in\ Liver\ Free\ fraction\ (\%) = \ (DPM_{Liver\ F}/DPM_{Total}) \times 100$$
$$Retention\ in\ Liver\ Bound\ fraction\ (\%) = \ (DPM_{Liver\ B}/DPM_{Total}) \times 100$$
$$Retention\ in\ Muscle\ Free\ fraction\ (\%) = \ (DPM_{Muscle\ F}/DPM_{Total}) \times 100$$
$$Retention\ in\ Muscle\ Bound\ fraction\ (\%) = \ (DPM_{Muscle\ B}/DPM_{Total}) \times 100$$
$$Catabolism\ (\%) = \ (DPM_{Traps}/DPM_{Total}) \times 100$$

Where DPM_{Total} is the sum of the radioactivity (DPM) found in the incubation seawater (DPM_{SW}), *Free* and *Bound* liver ($DPM_{Liver\ F}$ and $DPM_{Liver\ B}$) and muscle ($DPM_{Muscle\ F}$ and $DPM_{Muscle\ B}$) fractions, and CO_2 traps (DPM_{Traps}).

4.2.4. Chemical analysis

Dry matter, ash, crude protein (N x 6.25), crude lipid, gross energy and phosphorus contents were analysed following standard procedures of the Association of Official Analytical Chemists (AOAC, 2006) and were run in duplicates. Diets total amino acid profile was determined by ultra-high-performance liquid chromatography (UPLC), after acid hydrolysis. All analyses were performed as described by Teodósio et al. (2021b).

4.2.5. Data analysis

Data from the metabolic flux assays are presented as mean ± standard deviation. Data expressed as a percentage were arcsine square root transformed previously to the statistical analysis (Ennos, 2012). After being checked for normal distribution and homogeneity of variances, all data was analysed by two-way analysis of variance (ANOVA). Differences among amino acids metabolic fate as a function of dietary P/E ratio were identified by Tukey's multiple-comparison test at $P < 0.05$ level of significance. Statistical analyses were performed using the IBM SPSS Statistics 26 software.

Additionally, metabolic flux assays data were subjected to a principal component analysis (PCA) to verify differences between amino acids and diet formulations and find potential clusters of observations. PCA was carried out using the standard prcomp R function in the auto-scaled matrices. Score plots were generated for the two first principal components (PC1 and PC2) using the ggbiplot package for R. Loadings for PC1 and PC2 were calculated to determine the weight of each original variable in the corresponding PCs. Analyses were carried out using the open-source software R version 4.0.4.

4.3. RESULTS

The proportion of the tube-fed ^{14}C-amino acids evacuated was influenced by the P/E ratios (Figure 4.1). Feeding gilthead seabream juveniles with the HighP/E diet significantly increased the evacuation of amino acids when compared with LowP/E fed fish. Evacuation of protein and lysine in fish fed the HighP/E diet increased from 28% to 35% and from 24% to 47%, respectively, compared with LowP/E fed fish. Independently of the diet, methionine was significantly more evacuated (~ 46%) than protein while lysine presented intermediate results.

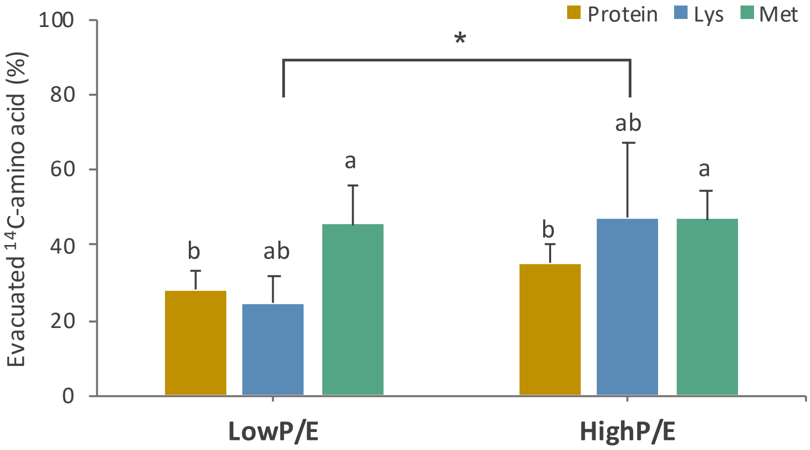

Figure 4.1 Proportion (%) of the total tube-fed ^{14}C-labelled protein (amino acid mixture), lysine (Lys) and methionine (Met) that was evacuated in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean ± standard deviation ($n = 6$ fish for each diet and tracer). Letters (a, b) represent significant differences in the metabolic fate of the distinct amino acids at each dietary treatment, and * denotes significant differences between dietary treatments ($p < 0.05$).

In the *Free* and *Bound* fractions of the liver, amino acid retention was not affected by the dietary treatment (Figure 4.2). Lysine was significantly more retained in the *Free* fraction than protein, while methionine presented intermediate values (Figure 4.2A). However, in the hepatic *Bound* fraction methionine was significantly less retained than protein or lysine, which presented similar retention levels (Figure 4.2B).

The dietary P/E ratio influenced the retention of amino acids in the *Free* fraction of the muscle (Figure 4.3A). A significantly higher proportion of ^{14}C-amino acids was found in the *Free* muscle fraction of fish fed the LowP/E diet compared with the HighP/E fed fish. A significantly higher percentage of the tube-fed ^{14}C-Met was found in this fraction compared with ^{14}C-protein or ^{14}C-Lys, with average values of 19%, 6% and 5%, respectively. No significant differences were found in the *Bound* muscle fraction of fish (Figure 4.3B).

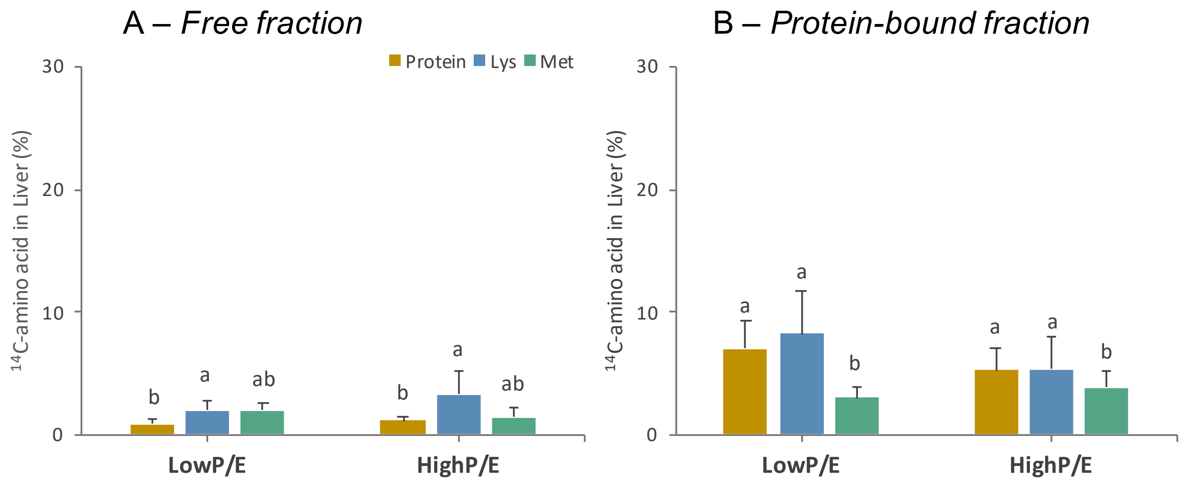

Figure 4.2 Proportion (%) of the total tube-fed ^{14}C-labelled protein (amino acid mixture), lysine (Lys) and methionine (Met) that was retained in the *Free* **(A)** and *Bound* **(B)** fractions of liver in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean ± standard deviation (n = 6 fish for each diet and tracer). Significant differences ($p <$ 0.05) in the metabolic fate of the distinct amino acids at each dietary treatment are represented by letters (a, b).

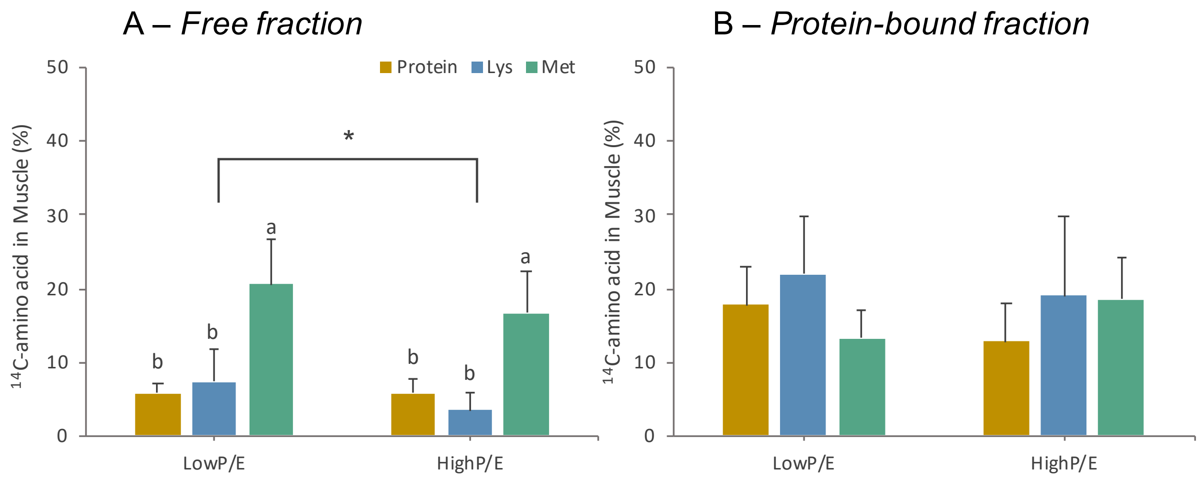

Figure 4.3 Proportion (%) of the total tube-fed ^{14}C-labelled protein (amino acid mixture), lysine (Lys) and methionine (Met) that was retained in the *Free* **(A)** and *Bound* **(B)** fractions of muscle in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean ± standard deviation (n = 6 fish for each diet and tracer). Letters (a, b) represent significant differences in the metabolic fate of the distinct amino acids at each dietary treatment, and * denotes significant differences between dietary treatments ($p < 0.05$). Absence of letters denote no significant differences.

Interacting effects between the metabolic fate of the distinct amino acids and the dietary treatments were found for amino acid catabolism (Figure 4.4). Catabolism of ^{14}C-protein remained unaltered in fish fed the LowP/E and HighP/E diets. However, lysine catabolism significantly decreased from 36% in fish fed the LowP/E diet to 22% in HighP/E fed fish. ^{14}C-Met was significantly less catabolised than ^{14}C-protein and ^{14}C-Lys in fish from both dietary treatments. Additionally, methionine catabolism did not differ significantly between dietary treatments.

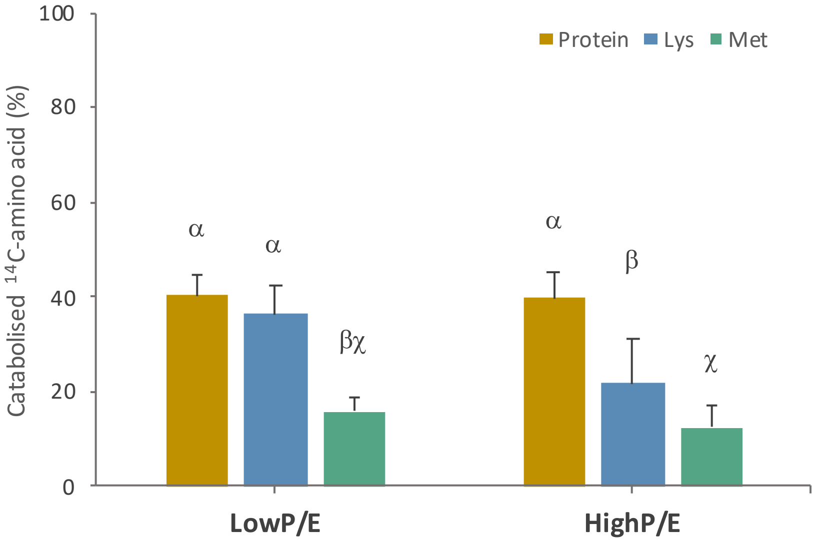

Figure 4.4 Proportion (%) of the total tube-fed ^{14}C-labelled protein (amino acid mixture), lysine (Lys) and methionine (Met) that was catabolised in gilthead seabream juveniles fed LowP/E or HighP/E diets. Values are presented as mean ± standard deviation ($n = 6$ fish for each diet and tracer). Interacting effects ($p < 0.05$) between the metabolic fate of the distinct amino acids and dietary treatments are represented by (α, β, χ).

Principal component analysis (PCA) was used to reduce the complexity of the data from the metabolic flux assays (Figure 4.5). PC1 and PC2 accounted for 45% and 25% of the total variability of the data. The analysis of the score plots indicated that methionine and protein data were separated along the PC1 as well as the PC2 axis. Evacuation, retention in the *Bound* fraction of liver and catabolism were the loadings that contributed the most for the dissimilarities observed between fish fed ^{14}C-protein labelled diets and fish fed diets labelled with ^{14}C-Met along the PC1 axis. Along the PC2 axis, catabolism, and retention in the *Free* fraction of liver and muscle, were responsible for the clustering of fish fed diets labelled with ^{14}C-protein and ^{14}C-Met in different groups.

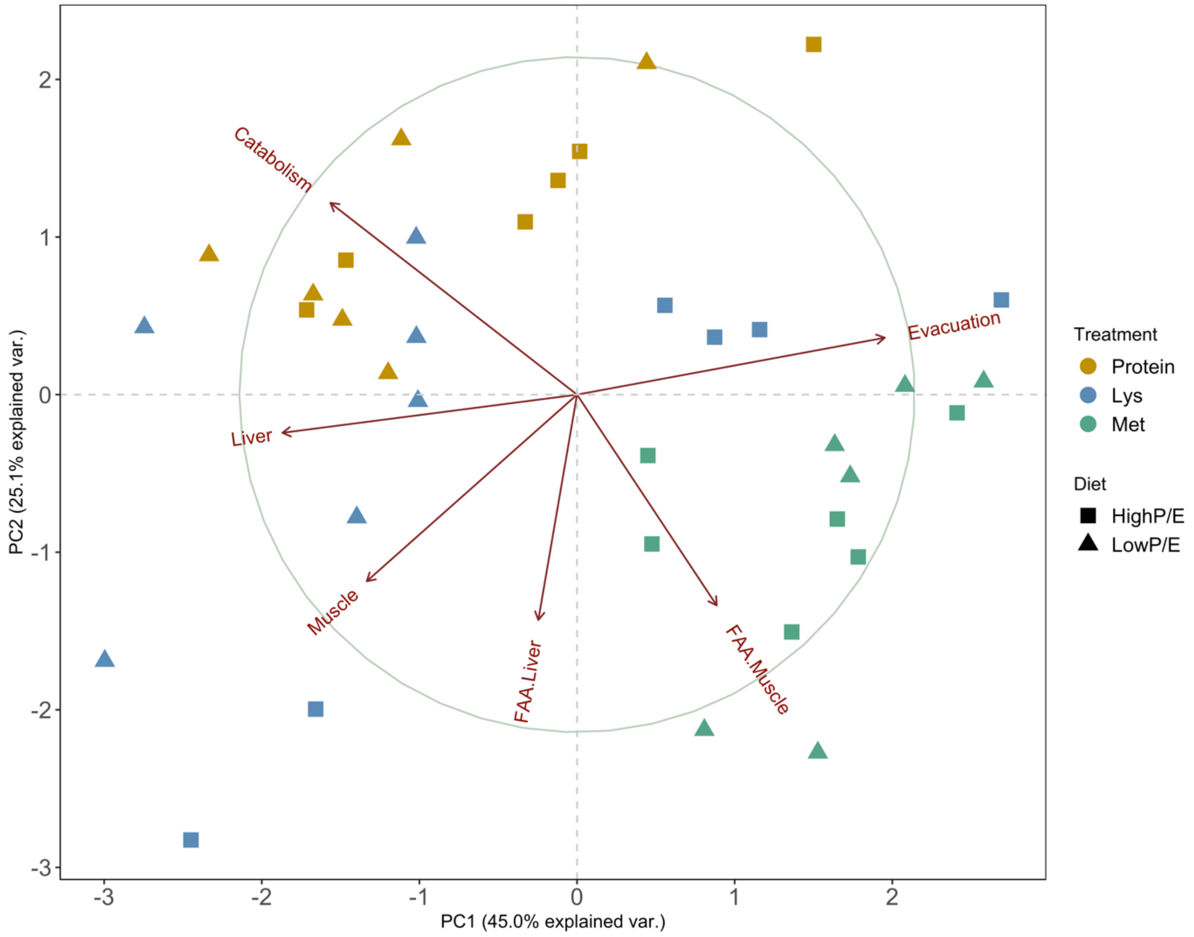

Figure 4. 5 Principal component analysis (PCA) of the metabolic flux assays data generated by gilthead seabream juveniles fed LowP/E and HighP/E diets labelled with ^{14}C-protein (amino acid mixture), lysine (Lys) and methionine (Met). Each point represents the projection of an individual sample in the PC1 and PC2 axis. Each dietary treatment is identified as a unique shape and each ^{14}C-amino acid as a unique colour, as indicated in the legend.

4.4. DISCUSSION

Metabolic flux assays were used to unravel the effect of dietary P/E ratios in the bioavailability and metabolic fate of protein, lysine, and methionine in gilthead seabream juveniles. In the present study, amino acid evacuation was influenced by the amino acids and dietary P/E ratios. Methionine was significantly more evacuated than protein, independently of the P/E ratios. Methionine evacuation (~ 46%) was similar to data observed in a previous study where metabolic flux assays were performed in gilthead seabream juveniles fed four different diets with distinct protein (44 and 40%) and/or lipid (21 and 18 %) levels (Chapter III). Evacuation of ^{14}C-protein (~ 30%) was in agreement with previous works that determined that the nutritional background (naïve or larvae exposed to a dietary glucose stimuli) did not influence protein utilisation in seabream juveniles (Rocha et al., 2016). PCA results confirmed that methionine and protein were clustered in different groups along the PC1 and PC2 axes mainly due to evacuation data, suggesting that methionine absorption is less efficient compared with most of the other protein-bound amino acids. Moreover, independently of the amino acid, significant differences in evacuation were found between fish fed the different diets. In contrast, in the abovementioned study in seabream fed diets with different protein and lipid levels, evacuation of lysine, tryptophan and methionine was not affected by the dietary formulations (Chapter III). On the other hand, the intestinal uptake of certain amino acids such as methionine, was shown to increase with a higher inclusion of dietary lipids in seabass fingerlings (García-Meilán et al., 2016). Accordingly, in the present study, a lower evacuation was observed with the decrease of dietary P/E ratio. A possible explanation for a lower evacuation in fish fed the LowP/E diet could be that an increase in dietary lipids may have led to a slower intestinal transit resulting in a more efficient amino acid absorption. The increase in dietary lipid content may overload the digestive capacity of fish, and consequently may delay gastric evacuation giving more time for the uptake of dietary lipids (Olsen and Ringø, 1997) and amino acids. Furthermore, the decrease in dietary P/E ratio has been shown to improve feed efficiency, nutrient retention, and protein conversion, indicating a better utilisation of dietary protein (Bureau et al., 2002; Company et al., 1999; Skalli et al., 2004; Thoman et al., 1999). A slower intestinal transit in fish fed high lipid diets (low dietary P/E ratio) may be one of the factors contributing to a more efficient amino acid uptake that could be translated into improved growth and protein utilisation.

In the current study, ^{14}C-amino acid retention in the hepatic *Free* and *Bound* fractions differed significantly among amino acids. Lysine was significantly more retained than protein in the former and than methionine in the latter. As previously suggested (Chapter III), the relatively high retention in liver, 18 h after feeding, may be related to amino acids essential role in energy homeostasis. However, the current study went further than the previous since the proportion of amino acids retained in the tissues was determined in the *Free* and *Bound* fractions and not only as a total. The protein-bound amino acids retained in the liver were higher than in the free pool in fish fed both diets. These findings reveal that protein synthesis is occurring in the liver, since the amino acids are being mostly incorporated into hepatic proteins within 18 h after feeding and not only temporarily available in the free pool.

The proportion of methionine found in the fish muscle, *i.e.* the sum of *Free* and *Bound* fractions, was higher than lysine or protein. In seabream fed diets with distinctive protein and/or lipid levels, total retention for methionine and lysine in the muscle was similar to the present results. Methionine was preferentially retained in the muscle, being more available for growth or other metabolic purposes (Chapter III). Similarly, in Senegalese sole larvae and juveniles and in white seabream larvae, most of the methionine was recovered in the muscle (Costas, 2011; Pinto et al., 2013; Rønnestad et al., 2000; Saavedra et al., 2008a). However, in fish fed both diets, no significant differences were found in the retention of amino acids in the muscle *Bound* fraction, while methionine retention was significantly higher than that of protein or lysine in the *Free* fraction. PCA results corroborate these findings since fish fed both diets labelled with ^{14}C-Met were grouped in a cluster according to data from the retention in the muscle *Free* fraction. These findings demonstrate that the higher retention of methionine in the muscle was due to its presence in the free pool and not as protein-bound, contrarily to what was observed for lysine. This reinforces the pivotal role of methionine in several metabolic functions beyond protein synthesis. Apart from protein synthesis, methionine participates in numerous metabolic reactions as a precursor of S-adenosylmethionine, cysteine, glutathione, taurine or polyamines (Brosnan and Brosnan, 2006; Wu, 2013). Moreover, this indispensable amino acid has been shown to be involved in the methylation of DNA and protein, cell proliferation and differentiation (Li et al., 2009; Wu, 2009). Lysine retention in the muscle was similar to protein, independently of the diet, confirming that lysine is a good indicator of muscle accretion. The amount of free amino acids retained in the muscle of fish fed the LowP/E diet was significantly higher than in the HighP/E fed fish. This change was due to a higher retention of lysine and methionine since the retention of ^{14}C-protein remained relatively unaltered. These results imply that a decrease in the dietary P/E ratios affect amino acid utilisation and may favour the retention of indispensable over dispensable amino acids in the muscle.

A fraction of the absorbed amino acids is unavoidably catabolised and used for energy rather than protein synthesis (Halver and Hardy, 2003). In a preceding work in gilthead seabream fed diets with distinctive levels of protein and/or lipids, catabolism was found to be predominantly ketogenic considering that lysine was the preferred amino acid to be used for energy even when deficient in the diet (Chapter III). The current work corroborates these findings since, irrespectively of the diet, lysine was significantly more catabolised than methionine. Catabolism was influenced by the interaction between the metabolic fate of the distinct amino acids and the P/E ratios, but although the catabolism of protein and methionine remained reasonably unaltered in fish fed both diets, this was not the case for lysine. Based on data from the catabolism of fish fed both diets labelled with ^{14}C-protein and ^{14}C-Met, PCA results demonstrate that protein and methionine were clustered in two different groups along the PC2 axis. Determination of the metabolic flux of protein revealed that the proportion of amino acids catabolised was approximately 40% of the ^{14}C-protein, independently of the diet.

The increase of methionine availability in the muscle *Free* fraction with a decrease in dietary P/E ratio was not translated into a higher catabolism. Methionine was the least catabolised (13-16%) amino acid, indicating that it is preferentially spared for other metabolic purposes instead of being used for energy. In contrast, it appears that a higher availability of free lysine resulted in increased catabolism in fish fed the LowP/E diet. While fish fed the LowP/E diet presented values as high as the ones for protein, fish fed the HighP/E diet drastically reduced lysine catabolism from 36 to 22%. Fish are able to selectively retain and catabolise distinct amino acids (Aragão et al., 2004; Saavedra et al., 2008a; 2008b; Chapter III). For instance, Senegalese sole postlarvae were found to use a significant higher proportion of the dispensable amino acids glutamate and alanine for energy, rather than the indispensable amino acids lysine and arginine, sparing these for growth (Rønnestad et al., 2001b). The current findings suggest that although fish fed both diets were equally using amino acids for energy, the dietary P/E ratios influenced which amino acids were catabolised. An increase in the P/E ratio in seabream diets reduces lysine catabolism and may channel other amino acids (*e.g.* dispensable amino acids) for energy, increasing lysine availability for growth or other metabolic functions.

4.5. CONCLUSION

Maximisation of fish growth while reducing nitrogen losses to the environment is strongly dependent on improving amino acid utilisation. This study revealed that a decrease in the P/E ratio by increasing the dietary lipid content resulted in lower amino acid evacuation. Methionine was the most retained amino acid in the muscle free pool, demonstrating its importance for metabolic functions other than protein synthesis. Moreover, increasing the dietary P/E ratio may spare lysine for growth or other metabolic purposes. An optimisation of P/E ratios in diets for gilthead seabream juveniles will improve diet utilisation and fish performance.

4.6. ACKNOWLEDGEMENTS

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Chapter V

Metabolic and nutritional responses of Nile tilapia juveniles to dietary methionine sources

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Metabolic and nutritional responses of Nile tilapia juveniles to dietary methionine sources

Abstract

Commercial diets for tilapia juveniles contain high levels of plant protein sources. Soybean meal has been utilised due to its high protein content; however, soy-based diets are limited in methionine and require its supplementation to fulfil fish requirements. DL-methionine (DL-Met) and calcium bis-methionine hydroxyl analogue (MHA-Ca) are synthetic methionine sources supplemented in aquafeeds, which may differ in biological efficiency due to structural differences. The present study evaluated the effect of both methionine sources on metabolism and growth of Nile tilapia. A growth trial was performed using three isonitrogenous and isoenergetic diets, containing plant ingredients as protein sources: DLM and MHA diets were supplemented on equimolar levels of methionine, while REF diet was not supplemented. Hepatic free methionine and one-carbon metabolites were determined in fish fed for 57 days. Metabolism of DL-Met and MHA was analysed by an *in vivo* time-course trial using ^{14}C-labelled tracers. Only DL-Met supplementation significantly increased final body weight and improved feed conversion and protein efficiency ratios compared to the REF diet. Our findings indicate that methionine in DLM fed fish follows the transsulfuration pathway while in fish fed MHA and REF diets it is remethylated. The *in vivo* trial revealed that ^{14}C-DL-Met is absorbed faster and more retained than ^{14}C-MHA, resulting in a greater availability of free methionine in the tissues when fish is fed with DLM diet. Our study indicates that dietary DL-Met supplementation improves growth performance and nitrogen retention, and that methionine absorption and utilisation is influenced by the dietary source in tilapia juveniles.

Keywords: Nile tilapia; Methionine sources; Methionine metabolism; Methionine cycle.

5.1. INTRODUCTION

Tilapia is the second most farmed fish worldwide, just after carp (FAO, 2020). Nevertheless, there are still many challenges to overcome in order to farm tilapia under economic and environmentally sustainable principles. Part of the challenge is dependent on the development of sustainable feeds that will ensure an optimal nutritional status for the fish while promoting nitrogen and phosphorus retention, thus reducing nutrient excretion into the aquatic environment.

In order to increase the sustainability of aquaculture feeds, inclusion levels of plant ingredients in fish diets have increased. Soybean meal has been successfully included in aquafeeds (Carter and Hauler, 2000; Glencross et al., 2004; Nguyen et al., 2009) due to its high protein content and relatively well-balanced amino acid profile. However, soy-based diets are limited in methionine and require its supplementation in order to fulfil fish requirements (Gatlin et al., 2007).

Methionine (Met) is an indispensable amino acid involved in protein synthesis, transmethylation reactions and antioxidant defence. Methionine metabolism occurs mainly in the liver where L-methionine is converted into S-adenosylmethionine (SAM), a methyl donor for several reactions. Subsequently SAM donates its methyl group and is transformed into S-adenosylhomocysteine (SAH), which is rapidly converted into homocysteine. Hepatic homocysteine can be remethylated back to methionine by adding a methyl group from trimethylglycine or can irreversibly enter the transsulfuration pathway (Selhub, 1999). The transsulfuration pathway converts homocysteine into cystathionine and then cysteine. Ultimately, cysteine can be incorporated into proteins, metabolised into glutathione or oxidised to form taurine (Stipanuk, 2004).

DL-methionine (DL-Met) and methionine hydroxyl analogue (MHA; DL-2-hydroxy-4-methylthiobutyrate or DL-HMTBA and its calcium salt) are synthetic sources of methionine that are often supplemented to animal feeds. DL-Met is a racemic mixture of D- and L-isomers of methionine, while MHA is a racemic mixture of D- and L-isomers of methionine hydroxyl analogue (Dibner, 2003). MHA chemical structure is similar to that of methionine however, it contains a hydroxyl group instead of the amino group. Recently, studies in rainbow trout (*Oncorhynchus mykiss*) suggested that DL-Met and MHA uptake in the gut apical surface is facilitated by sodium-dependent transporters and mediated by proton-independent transporters across the basolateral membrane (To et al., 2019; 2020). Data comparing the intestinal flux rates of DL-Met and MHA suggests a faster intestinal transport of the former synthetic source (Dibner, 2003; Maenz and Engele-Schaan, 1996a; To et al., 2020).

Since animals can only metabolise L-amino acids, D-isomers of DL-Met first need to be converted to a keto-methionine intermediate (keto-methylthio-butanoic acid, KMB) and then transaminated to L-Met before becoming available (Dibner, 2003; Zhang et al., 2015). On the other hand, both the D- and L-isomers of MHA need to be converted to KMB to become available (Dibner, 2003; Zhang et al., 2015). These differences are likely to be reflected as differences in absorption and metabolism and may result in different biological efficiencies. Numerous studies in terrestrial animals and fish advocate that MHA supplementation results in lower bioavailability of this compound compared to DL-Met (Keembiyehetty and Gatlin, 1997; Kelly et al., 2006; Sauer et al., 2008). In rainbow trout, dietary methionine hydroxyl analogue calcium salt (MHA-Ca) was found to have lower bioavailability than DL-Met, resulting in 69% lower fish weight gain, 60% lower growth rate and 73% lower nitrogen retention in fish (Powell, et al., 2017). Similarly, growth performance and feed utilisation indicators in common carp (*Cyprinus carpio*) fed DL-Met and MHA-Ca supplemented diets, demonstrated that MHA-Ca was 41% to 50% as available as DL-Met on weight-for-weight basis (Zhou et al., 2021). In contrast, some authors report similar efficiencies among methionine sources based on growth and feed efficiency in several aquatic animals (Forster and Dominy, 2006; Goff and Gatlin, 2004; Pan et al., 2016; Zhou et al., 2018). On the other hand, a study performed in channel catfish observed that dietary MHA-Ca supplementation resulted in improved body weight, weight gain, feed conversion ratio and protein efficiency ratio (Zhao et al., 2017). Based on the data published, NRC (2011) concluded that it is reasonable to assume that the biological efficacy of MHA in fish is 75-80% that of DL-Met on an equimolar basis (63 – 67% on a weight basis). Generating data on growth, diet utilisation and methionine metabolism in a commercial relevant species such as the Nile tilapia (*Oreochromis niloticus*) is of paramount importance for the aquaculture industry.

In this context, the objective of this study was to understand how the dietary source of methionine affects methionine metabolism and growth of Nile tilapia juveniles. Radiolabelled methionine sources were used in a nutrient flux assay to evaluate their influence in the amino acid metabolic pathways.

5.2. MATERIAL AND METHODS

5.2.1. Diets

Three experimental diets were formulated to be isonitrogenous and isoenergetic (Table 5.1). Diet REF was a negative control, without fishmeal inclusion, formulated to be 40% below the methionine requirement for Nile tilapia (NRC, 2011). No methionine was supplemented to this diet. Experimental diets were the REF diet supplemented with 0.15% DL-methionine (DLM diet) and 0.18% calcium bis-methionine hydroxyl analogue (equal on molar basis to 0.15% DL-methionine; MHA diet). To minimise variability among diets, one common basal diet was formulated, and the respective supplemental methionine source was added in DLM and MHA diets. Diets were formulated to meet the minimum requirements of amino acids on digestible basis for Nile tilapia juveniles, except for methionine. Apparent digestibility coefficients (ADC) of amino acids for the ingredients used were taken from published review data (Konnert and Masagounder, 2017; Konnert et al., 2017). Diets were supplemented with selected indispensable amino acids and di-calcium phosphate to avoid amino acid or mineral imbalances.

Upon ingredient grinding with a hammer mill (model SH1, Hosokawa-Alpine, Germany) and its mixing in a double-helixmixer, all diets (pellet sizes 1.2 and 2.0 mm) were manufactured using a twin-screw extruder (model BC45, Clextral, France) at SPAROS Lda. (Olhão, Portugal). Upon cold extrusion, diets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, the oils were added to the pellets by vacuum coating (model PG-10VCLAB, Dinnisen, The Netherlands). Throughout the duration of the trial, experimental feeds were stored at room temperature, in a cool and aerated storage room. Proximate composition and amino acid analysis were determined in all experimental diets, as reported in Tables 5.1 and 5.2, respectively.

TABLE 5.1 Formulation and proximate composition of the experimental diets.

	Dietary treatments		
Ingredients (%)	**REF**	**DLM**	**MHA**
Soybean meal [a]	35.00	34.95	34.94
Soy protein concentrate [b]	9.25	9.24	9.23
Corn meal [c]	29.92	29.87	29.86
Pea protein concentrate [d]	7.78	7.76	7.76
Wheat bran [e]	6.63	6.62	6.62
Soybean oil [f]	4.80	4.79	4.79
Fish oil [g]	2.00	2.00	2.00
Di-calcium phosphate [h]	3.00	3.00	2.99
Vit-Min premix [i]	1.00	1.00	1.00
L-Lysine sulfate	0.31	0.31	0.31
L-Threonine	0.19	0.19	0.19
L-Tryptophan	0.06	0.06	0.06
L-Histidine	0.06	0.06	0.06
DL-Methionine	0.00	0.15	0.00
Calcium bis-methionine hydroxyl analogue	0.00	0.00	0.18
Analysed proximate composition (as fed basis)			
Dry matter	94.16	91.97	95.05
Ash	5.83	6.12	6.28
Crude protein	32.64	32.31	32.39
Crude fat	8.82	8.93	9.53
Total phosphorus	0.73	0.79	0.81
Gross energy (MJ kg^{-1})	19.0	18.6	19.2

[a] Solvent extracted dehulled soybean meal: 45.7% crude protein (CP), 3.1% crude fat (CF), CARGILL, Spain.
[b] Soycomil P: 62.0% CP, 0.7% CF, ADM, The Netherlands.
[c] Corn meal: 8.1% CP; 3.7% CF, Casa Lanchinha, Portugal.
[d] Lysamine GPS: 84.0% CP, 1.0% CF, ROQUETTE Frères, France.
[e] Wheat bran: 14.9% CP, 4.0% CF, Cerealis Moagens S.A., Portugal.
[f] Henry Lamotte Oils GmbH, Germany.

[g] Sopropêche, France.

[h] DCP: 16.8% phosphorus, 20.9% calcium, Premix Lda, Italy.

[i] PREMIX Lda, Portugal. Vitamins (mg or mg kg^{-1} diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 6.88 mg; DL-cholecalciferol, 50 mg; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg kg^{-1} diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.

TABLE 5.2 Amino acid and calcium bis-methionine hydroxyl analogue (MHA-Ca) content of experimental diets.

	Dietary treatments		
Analysed values (mg g^{-1} diet)	**REF**	**DLM**	**MHA**
Lysine	17.8	19.7	19.1
Methionine	4.4	5.6[*]	4.4
Cysteine	4.4	4.3	4.4
Threonine	13.5	13.6	13.8
Arginine	23.0	23.3	23.9
Isoleucine	13.9	14.0	14.3
Leucine	25.2	25.2	25.7
Valine	15.2	15.2	15.7
Histidine	8.3	8.5	8.7
Phenylalanine	15.9	16.0	16.3
Glycine	13.3	13.3	13.7
Serine	16.0	15.7	16.2
Proline	17.1	16.7	17.1
Alanine	14.4	14.3	14.7
Aspartate	34.2	34.4	35.4
Glutamate	56.7	55.9	57.3
MHA-Ca[†]	0.0	0.0	1.3
Taurine	< 0.1	< 0.1	< 0.1

[*] Analysed DL-Met in the DLM diet was 1.1 mg g^{-1} diet.

[†] Active content of MHA in the MHA diet is 1.1 mg g^{-1} (equal on molar basis to DL-Met) since MHA-Ca active form is 84 %.

5.2.2. Growth trial

The experiment was carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Nile tilapia (Silver Natural Male Tilapia™) juveniles were obtained from Til-Aqua International B.V. (The Netherlands) and the experiment was conducted at Centre of Marine Sciences of Algarve (CCMAR) facilities (Faro, Portugal). CCMAR facilities and their staff are certified to house and conduct experiments with live animals (Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal). Upon arrival, fish were acclimatised to the new rearing facilities in a recirculating aquaculture system and were fed a commercial diet (crude protein: 34%; crude fat: 5%).

Juvenile Nile tilapia were reared in 100 L cylindrical tanks in a recirculating aquaculture system equipped with a mechanical filter, a submerged biological filter and a UV steriliser. Photoperiod was natural (10 h light: 14 h dark), temperature averaged 25.9 ± 0.5°C and dissolved oxygen in water was maintained above 85% of saturation. Water quality parameters were monitored daily and adjusted when necessary: pH was maintained between 7.0 and 8.2 and the concentration of unionized ammonia and nitrites in water was 0 mg L^{-1} during the whole experimental period. Mortality was monitored daily.

Fish with an initial mean body weight of 2.3 ± 0.4 g were allocated into nine tanks at an initial density of 1.1 kg m^{-3} (50 fish per tank). Eight fish from the initial stock were sampled and the whole fish were stored at -20 ºC until analysis of proximate composition. Triplicate tanks were randomly assigned to one of the three dietary treatments (REF, DLM and MHA). Fish were fed to visual satiety by hand, three times a day (09.30, 12.30 and 16.30 hours) and feed intake was recorded daily for 57 days.

At the end of the trial, each tank was bulk weighed. Ten fish from each tank were euthanised with a lethal dose of anaesthetic (1.5 mL L^{-1} phenoxyethanol, Sigma-Aldrich, Spain). Whole-body, liver and viscera weight of five individual fish were recorded for calculation of biometric indexes and liver samples were snap-frozen in liquid nitrogen and kept at –20°C until free amino acid analysis. The other five fish were stored at –20°C until analysis of whole-body proximate composition and amino acid content. Fish were fasted for 24 h before initial and final samplings.

5.2.3. Metabolic utilisation of supplemental methionine sources

At the end of the growth trial, to understand how different dietary methionine sources are absorbed and metabolised in tilapia juveniles, a time-course metabolic trial was performed using radiolabelled DL-Met and MHA. REF diet was not included in this trial since the aim was to assess putative differences in the metabolic flux of the methionine supplemental sources and not of the intact protein. Random fish fed the DLM or MHA diet were transferred to the nutrient flux laboratory after being fasted for 24 h.

DL-(1-^{14}C)-methionine (^{14}C-DL-Met; 0.00185 GBq) and (1-^{14}C)-calcium bis-methionine hydroxyl analogue (^{14}C-MHA; 0.00185 GBq) (Campro Scientific GmbH, The Netherlands) were used as tracers to radiolabel the experimental diets (DLM and MHA). The methodology for labelling the experimental diets was established in previous works of the authors (Richard et al., 2017; Rocha et al., 2016a; Teodósio et al., 2020). The tracers were diluted in freshwater Ringer solution and a known value of the tracer was dispensed using a micropipette on individual pellets of the correspondent experimental diet. The pellets were dried at 50°C for 1 h. Eight pellets per fish, corresponding to 0.3% body weight, were loaded into a hollow plastic tube of 1.5 mm inner diameter, and stored for subsequent tube-feeding. Prior to tube-feeding, the amount of radioactivity (disintegrations per minute; DPM) of ten individual pellets from each experimental diet labelled with the corresponding tracer was determined in a TriCarb 2910TR low activity liquid scintillation analyser (Perkin Elmer, USA) after adding the scintillation cocktail (Ultima Gold XR, Perkin Elmer).

The *in vivo* method of tube-feeding used to perform the metabolic trials was adapted from Costas (2011), which was a modification of the method first described by Rust et al. (1993) and modified by Rønnestad et al. (2001). Fish were anaesthetised (200 mg L^{-1} MS-222 buffered with sodium bicarbonate, Sigma-Aldrich) and subsequently were taken out of the water using a fish net and placed onto a dry plastic tray. The previously loaded plastic tube containing the radiolabelled pellets was inserted into the fish mouth and the feed pellets were gently pushed directly into the oesophagus using a solid piece with a smaller diameter placed inside as a plunger. The diameter and length of the hollow plastic tube was previously tested to avoid injuring the fish oesophagus. This procedure lasted approximately ten seconds. The metabolic fate of the tracer (^{14}C-DL-Met or ^{14}C-MHA) was considered to represent the fate of the tracee (DL-Met or MHA-Ca) (Conceição et al., 2007).

After tube-feeding, fish were placed into a tank with clean and aerated freshwater to eliminate any residual anaesthetic and were monitored for eventual pellet regurgitation. After this period, fish were transferred into individual incubation chambers containing 2 L of freshwater at 26°C. Each chamber was hermetically sealed and supplied with a gentle oxygen flow. After the incubation period, oxygen flow was stopped and fish were euthanised inside the chambers by a lethal dose of anaesthetic (750 mg L^{-1} of MS-222 buffered with sodium bicarbonate). The incubation periods were 1, 2, 3, 4 or 6 h ($n = 6 – 7$ fish for each diet and incubation period). Fish was removed from the chamber and weighed.

Water samples were collected from each individual chamber to determine the amount of radioactivity (DPM) present in the incubation water. The radioactivity present in the incubation water resulted from evacuated (non-absorbed) and/or catabolised radiolabelled methionine source as CO_2. Viscera, liver, skin-on fillets and the rest of the fish were collected and weighed. Viscera consisted of washed digestive tract (so that no alimentary bolus was present), spleen, pancreas and perivisceral fat and will be designated from hereafter as *Viscera* compartment; skin-on fillets (muscle with skin), as *Muscle* compartment; and the rest of the fish (consisting of head, heart, kidney, bones, and fins) as *Residual* compartment. *Viscera* and *Liver* compartments were analysed as whole. *Muscle* and *Residual* compartments were minced using a coffee grinder until a homogeneous mixture was obtained and 0.5 g samples were taken for further analysis.

All fish tissues were incubated at 4°C for 24 h with 6% (w/v) trichloroacetic acid (TCA), with periodical stirrings. After this period, tissue samples were taken and the TCA samples collected for radioactivity determination (from hereafter designated as *Free* fraction). In order to get a better insight of the metabolic flux of ^{14}C-DL-Met and ^{14}C-MHA, tissue samples from the 6 h incubation period were homogenised and underwent a series of extraction procedures to separate organic compounds such as protein, lipids, and other metabolites as described previously by Rocha et al. (2016a). Briefly, samples were transferred to a clean vial and further homogenised in distilled water using an Ultra-turrax homogeniser (IKA, Germany). Total lipids were extracted using a modified Bligh and Dyer method (Bligh and Dyer, 1959) for small volumes and total protein was extracted based on a TCA precipitation method (Panchout et al., 2013). The supernatant containing non-extracted metabolites was collected for radioactivity determination. Protein pellet was resuspended in an appropriate volume of Solvable™ (Perkin Elmer, USA) and kept at 50ºC until complete solubilisation was achieved. Lipids and other metabolites are designated as *Others* fraction, while protein as *Protein* fraction. Scintillation cocktail (Ultima Gold XR, Perkin Elmer) was added to all samples and DPM were counted in a TriCarb 2910TR low activity liquid scintillation analyser (Perkin Elmer). All counts were corrected for quench and lumex. Radioactivity found in the *Incubation Water* and in the *Free* fractions of all compartments was normalised for fish or tissue weight and expressed as DPM per gram. Radioactivity present 6 h after feeding the radiolabelled nutrients in the *Protein* and *Others* fractions of all compartments was expressed as DPM.

To estimate the availability of the radiolabelled nutrient in each compartment, the area under the curve from 1 to 6 h was calculated as follows:

$$\sum_{i=1}^{n-1}\left[\left((DPM_{i+1} + DPM_i) \times (t_{i+1} - t_i)\right)/2\right]$$

where t is the time point, DPM_i the amount of radioactivity found at t_i and n the total number of measures (Pruessner et al., 2003). Area under the curve was expressed as a percentage of total cumulated (1 to 6 h) radioactivity (DPM) per dietary treatment.

5.2.4. Biochemical analysis

Raw materials (soybean meal, soy protein concentrate, pea protein concentrate, corn meal and wheat bran) were analysed for dry matter, crude protein and amino acid content using NIR (AMINONIR®, Evonik Nutrition & Care, Germany) before diet formulation.

Chemical analysis followed standard procedures of the Association of Official Analytical Chemists (AOAC, 2006) and were run in duplicates. Before analysis, diets and pooled whole-body fish were finely ground. Moisture content was determined by drying the samples at 105°C for 24 h and ash content by incineration in a muffle furnace at 550°C for 6 h. Freeze-dried whole-body samples and diets were analysed for crude protein (N x 6.25) by wet chemistry (AMINOLab®, Evonik Nutrition & Care) using the combustion/Dumas method; crude fat by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany); gross energy by combustion in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany) calibrated with benzoic acid and phosphorus content by digestion at 230°C in a Kjeldatherm block digestion unit followed by digestion at 75°C in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04-406).

Amino acid content in diets and whole-body fish samples was analysed by wet chemistry (AMINOLab®, Evonik Nutrition & Care) using ion exchange chromatography. Hepatic free amino acids, SAM and SAH contents were determined after homogenisation of freeze-dried samples in 0.1 M HCl on ice, centrifugation at 1500 x *g* at 4°C for 15 min and deproteinization of the supernatant by centrifugal ultrafiltration (10 kDa cut-off, 2500 x *g* at 4°C for 20 min). All samples were pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA), except samples for SAM and SAH analysis, which were not derivatised. All analyses were performed by ultra-high-performance liquid chromatography (UPLC) on a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Amino acids were identified by retention times of standard mixtures (Waters) and pure standards (Sigma-Aldrich). Instrument control, data acquisition and processing were achieved by the use of Waters Empower software.

5.2.5. Nutritional indicators

Growth performance parameters, somatic indexes and nutrient retention were calculated as follows:

Daily voluntary feed intake (VFI, % day^{-1}) = 100 × apparent feed intake × ABM $^{-1}$ × days^{-1}, where ABM is average body mass = (final biomass + initial biomass)/2.

Feed conversion ratio (FCR) = apparent feed intake × wet weight gain^{-1}.

Protein efficiency ratio (PER) = wet weight gain × crude protein intake^{-1}.

Hepatosomatic index (HSI %) = 100 × liver weight × total weight^{-1}.

Viscerosomatic index (VSI %) = 100 × viscera weight × total weight^{-1}.

Protein or energy retention (% intake) = 100 × [(final body protein or energy content – initial body protein or energy content) × (protein or energy intake)$^{-1}$].

Daily nitrogen intake (mg N kg^{-1} day^{-1}) = nitrogen intake × ABM^{-1} × days^{-1}.

Daily nitrogen gain (mg N kg^{-1} day^{-1}) = (final body nitrogen content – initial body nitrogen content) × ABM^{-1} × days^{-1}.

Daily nitrogen loss (mg N kg^{-1} day^{-1}) = daily nitrogen intake – daily nitrogen gain.

5.2.6. Statistical analysis

Sample size was determined based on preliminary power analysis to ensure a probability of at least 80% in the detection of treatment effects. Data are presented as means ± standard deviation. Data expressed as a percentage were arcsine square root transformed previously to the statistical analysis (Ennos, 2012). All data were checked for normal distribution and homogeneity of variances. Differences among groups were identified by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test or, when the assumptions for the ANOVA failed, by Kruskal-Wallis one-way analysis of variance by ranks followed by Dunn's multiple-comparison tests. Data from the time-course metabolic trials were subjected to linear regression analysis to understand the relationship between the tracer (^{14}C-DL-Met or

^{14}C-MHA) and incubation time in each compartment (*Incubation Water*, *Viscera*, *Liver*, *Muscle* and *Residual* compartments). Additionally, one-way ANOVA followed by planned contrasts test was performed to compare the differences between ^{14}C-DL-Met and ^{14}C-MHA fed fish at each time point. At 6 h time point, Mann-Whitney *U* test was additionally used to identify differences in the *Protein* and *Others* fractions of the several body compartments. All statistical differences were considered significant at $P < 0.05$. Statistical analyses were performed using the open-source software R version 3.6.1.

5.3. RESULTS

5.3.1. Growth performance and feed utilisation

At the end of the growth trial, survival was 100% in all treatments and fish had a 9 to 10-fold increase in body weight (Table 5.3). Fish fed the DLM diet were significantly heavier (28.3 ± 4.7 g) than fish fed the REF diet (24.1 ± 3.0 g) and the MHA (24.8 ± 2.7 g) diets ($p < 0.05$). Fish biomass gain differed marginally with the different dietary treatments ($p = 0.08$). Feed intake was similar among experimental groups. Fish fed the DLM diet presented a significantly lower feed conversion ratio (FCR) than fish fed the REF diet ($p < 0.05$). Protein efficiency ratio (PER) was significantly higher in fish fed the DLM (3.4 ± 0.1) than the REF (3.1 ± 0.1) diet ($p < 0.05$). Dietary treatments did not affect significantly hepatosomatic and viscerosomatic indexes ($p > 0.05$).

5.3.2. Whole-body fish composition and nutrient retention

Fish from the DLM diet presented significantly higher body protein and energy content than fish from the MHA diet ($p < 0.05$; Table 5.4). Fish whole-body moisture, ash, fat and phosphorus content were not affected by the dietary treatments ($p > 0.05$). Additionally, whole-body total methionine content was significantly higher in DLM fed fish (3.86 ± 0.01 mg g^{-1}) than in fish from the REF (3.54 ± 0.11 mg g^{-1}) or MHA (3.50 ± 0.14 mg g^{-1}) dietary treatments ($p < 0.05$). No significant differences were found for the other amino acids among experimental groups. Protein and energy retention were significantly higher for fish fed the DLM diet than for fish fed the other diets ($p < 0.05$; Table 5.4).

TABLE 5.3 Growth performance, somatic indexes and feed utilisation of Nile tilapia juveniles fed the experimental diets for 57 days.

	Dietary treatments		
	REF	**DLM**	**MHA**
Final body weight (g)	24.1 ± 3.0^{b}	28.3 ± 4.7^{a}	24.8 ± 2.7^{b}
Biomass gain (g/tank)	928 ± 85	1142 ± 55	1052 ± 118
Daily voluntary feed intake (% average biomass day^{-1})	2.8 ± 0.1	2.7 ± 0.1	2.7 ± 0.1
Feed conversion ratio (FCR)	1.0 ± 0.0^{a}	0.9 ± 0.0^{b}	0.9 ± 0.0^{ab}
Protein efficiency ratio (PER)	3.1 ± 0.1^{b}	3.4 ± 0.1^{a}	3.3 ± 0.1^{ab}
Hepatosomatic index (HSI, %)	1.53 ± 0.47	1.34 ± 0.33	1.30 ± 0.20
Viscerosomatic index (VSI, %)	9.47 ± 0.64	9.07 ± 1.02	8.74 ± 1.37

Initial body weight = 2.3 ± 0.4 g for all dietary treatments (n = 150).
Values are presented as means ± standard deviation (n = 15 for final body weight, HSI and VSI; n = 3 for the remaining parameters). Different superscripts within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences.

TABLE 5.4 Whole-body composition and protein and energy retention of Nile tilapia juveniles fed the experimental diets for 57 days.

	Dietary treatments		
Body composition (% wet weight)	**REF**	**DLM**	**MHA**
Moisture	71.72 ± 0.28	70.98 ± 0.31	72.58 ± 1.26
Ash	3.63 ± 0.23	3.83 ± 0.05	3.58 ± 0.05
Protein	14.78 ± 0.33^{ab}	15.59 ± 0.07^{a}	14.51 ± 0.54^{b}
Fat	8.22 ± 0.33	7.94 ± 0.19	7.42 ± 0.41
Phosphorus	0.47 ± 0.02	0.51 ± 0.01	0.49 ± 0.02
Energy (MJ kg^{-1})	6.6 ± 0.1^{ab}	6.8 ± 0.1^{a}	6.3 ± 0.3^{b}
Retention (% intake)			
Protein	44.7 ± 2.2^{b}	52.2 ± 1.7^{a}	47.8 ± 0.8^{b}
Energy	35.7 ± 1.2^{b}	40.6 ± 1.4^{a}	36.1 ± 0.6^{b}

Initial body composition: moisture = 74.28% WW; ash = 4.00% WW; protein = 16.46% WW; fat = 4.77% WW; phosphorus = 0.59% WW; energy = 5.5 MJ kg^{-1} WW.

Values are presented as means ± standard deviation ($n = 3$). Different superscripts within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences.

Dietary treatments did not influence daily nitrogen intake. DLM fish exhibited the highest daily nitrogen gain (726 ± 3 mg N kg^{-1} day^{-1}), significantly different from REF (657 ± 8 mg N kg^{-1} day^{-1}) or MHA (660 ± 22 mg N kg^{-1} day^{-1}) fed fish ($p < 0.05$). Moreover, fish from the DLM group presented the lowest daily nitrogen loss (667 ± 43 mg N kg^{-1} day^{-1}), which was significantly different from the REF group ($p < 0.05$; Figure 5.1).

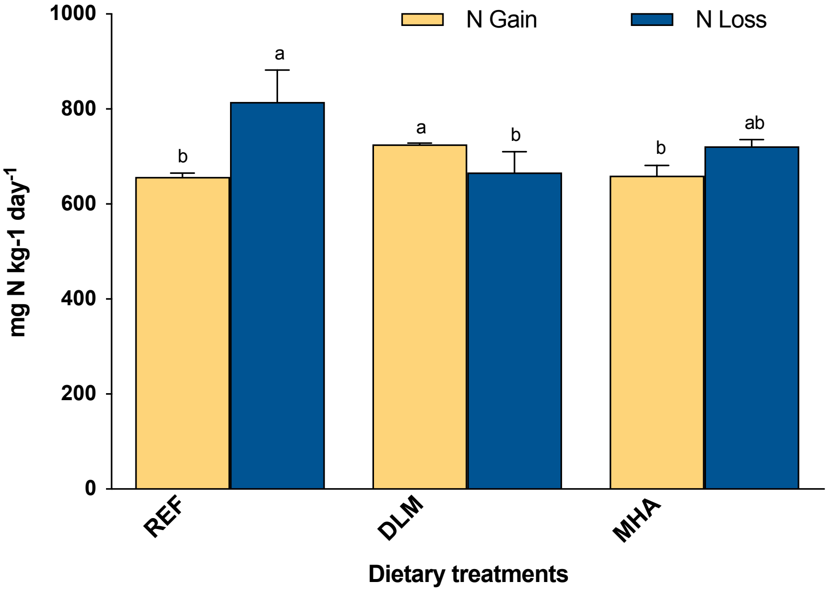

Figure 5.1 Daily nitrogen (N) balance in Nile tilapia juveniles fed the experimental diets for 57 days. Values are presented as means ± standard deviation ($n = 3$). Different letters within the same compartment indicate significant differences ($p < 0.05$) among diets.

5.3.3. Methionine and one-carbon metabolites

Free methionine (Figure 5.2A) was significantly higher in liver of fish fed the DLM and MHA diets than in REF fed fish ($p < 0.05$). S-adenosylmethionine (SAM; 1.25 to 1.35 mg SAM g^{-1} DW liver), S-adenosylhomocysteine (SAH; 0.23 to 0.27 mg SAH g^{-1} DW liver) or SAM/SAH ratio (4.79 to 5.48) were not affected by the dietary treatments ($p > 0.05$). Homocysteine levels (Figure 5.2B) were significantly lower in DLM fish than in fish fed REF and MHA diets ($p < 0.05$). Cystathionine was significantly lower in fish fed the supplemented diets than in fish fed the REF diet ($p < 0.05$; Figure 5.2C). Free cysteine content was not affected by the dietary treatments ($p > 0.05$; 0.17 to 0.20 mg Cys g^{-1} DW liver). Fish fed the DLM diet had more hepatic taurine content than fish fed REF and MHA diets ($p < 0.05$). In addition, there were no significant differences in taurine content between fish fed REF and MHA diets ($p > 0.05$; Figure 5.2D). Hepatic trimethylglycine was significantly lower in fish fed the DLM (0.05 ± 0.00 mg TMG g^{-1} DW liver) than the REF (0.06 ± 0.00 mg TMG g^{-1} DW liver) diet ($p < 0.05$). No significant differences in hepatic trimethylglycine content were found for fish fed the MHA diet compared to the other groups ($p > 0.05$).

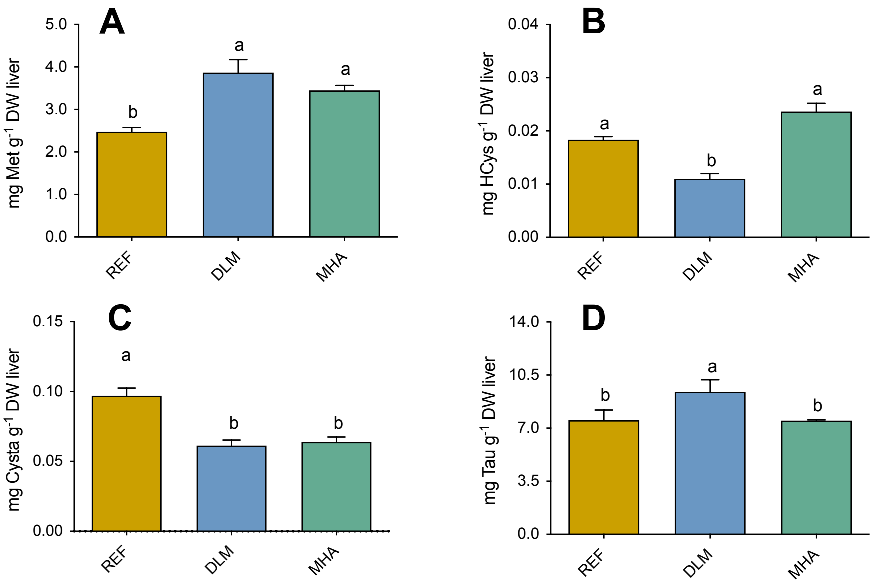

Figure 5.2 Free methionine (**A**), homocysteine (**B**), cystathionine (**C**) and taurine (**D**) content in liver of Nile tilapia juveniles fed the experimental diets for 57 days. Values are presented as means ± standard deviation ($n = 3$). Different letters indicate significant differences ($p < 0.05$) among diets.

Moreover, total free amino acids were significantly higher in DLM fish liver (108.42 ± 2.90 mg g^{-1} DW liver) than in liver of fish fed the REF (86.48 ± 3.29 mg g^{-1} DW liver) and the MHA (86.08 ± 1.81 mg g^{-1} DW liver) diets ($p < 0.05$). Similarly, the sum of free indispensable (IAA) and dispensable amino acids (DAA) were significantly higher in liver of fish fed the DLM diet than in liver of fish fed the other diets ($p < 0.05$; Figure 5.3).

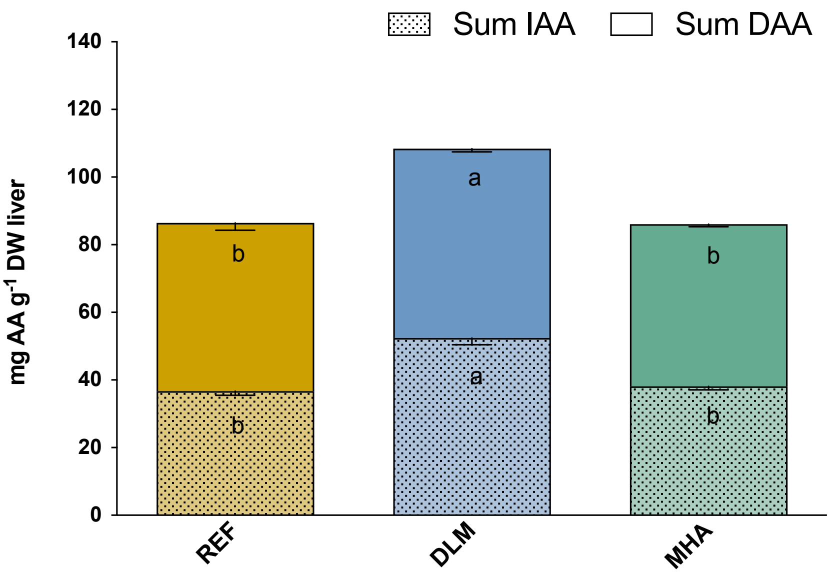

Figure 5.3 Sum of free indispensable (Sum IAA) and dispensable (Sum DAA) amino acids in liver of Nile tilapia juveniles fed the experimental diets for 57 days. Values are presented as means ± standard deviation (*n* = 3). Different letters within the same compartment indicate significant differences ($p < 0.05$) among diets.

5.3.4. Metabolic utilisation of supplemental methionine sources

The amount of radiolabelled tracer found in *Incubation Water* had a linear increase during the time-course for both DLM and MHA treatments (Table 5.5; $p < 0.05$). Also, at the end of the time-course (6 h) a higher amount of ^{14}C-MHA was present in the *Incubation Water* compartment than of ^{14}C-DL-Met ($p < 0.05$; Figure 5.4). Furthermore, the area under the curve for the MHA diet was 2.4-fold higher than for the DLM diet, indicating a higher evacuation and/or catabolism of ^{14}C-MHA compared to ^{14}C-DL-Met by juvenile tilapia (Table 5.5).

After 1 h of diet ingestion, the amount of tracer found in *Viscera* (Figure 5.5A) and *Liver* (Figure 5.5B) *Free* fractions was higher in the DLM than in the MHA fish, although only significantly different for the *Liver Free* fraction ($p < 0.05$; Figure 5.5B). The ^{14}C-DL-Met presented a linear decrease during the incubation period in both compartments ($p < 0.05$; Table 5.5). On the other hand, this linear pattern was not found in fish fed MHA diet. The amount of tracer in *Viscera Free* fraction increased up to 4 h and then decreased at 6 h to values slightly below the values determined for the DLM fed fish (Figure 5.5A). After 4 h of ingestion, the amount of tracer found in *Viscera Free* fraction of MHA fed fish was significantly higher than in fish fed the DLM diet ($p < 0.05$; Figure 5.5A). Regarding the ^{14}C-MHA in the *Liver Free* fraction, a plateau was observed from 2 h until the end of the incubation period (Figure 5.5B). The area under the curve in the *Viscera* and *Liver Free* fractions were similar for both dietary treatments, indicating a similar bioavailability of both methionine sources along the experimental period (Table 5.5).

TABLE 5.5 Linear regression analysis and area under the curve (1-6 h) of the free fractions analysed in the time-course metabolic trial.

Compartments	**DLM**	**MHA**
Linear regression p value		
Incubation Water	<0.001	<0.001
Viscera	0.015	0.489
Liver	0.010	0.222
Residual	0.219	0.002
Muscle	0.821	0.005
Area under the curve * ***(% total cumulated radioactivity DPM)***		
Incubation Water	2.0	4.7
Viscera	54.5	60.0
Liver	29.7	26.1
Residual	8.3	6.3
Muscle	5.5	2.9

[*] Please refer to *Metabolic utilisation of supplemental methionine sources* section for further details.

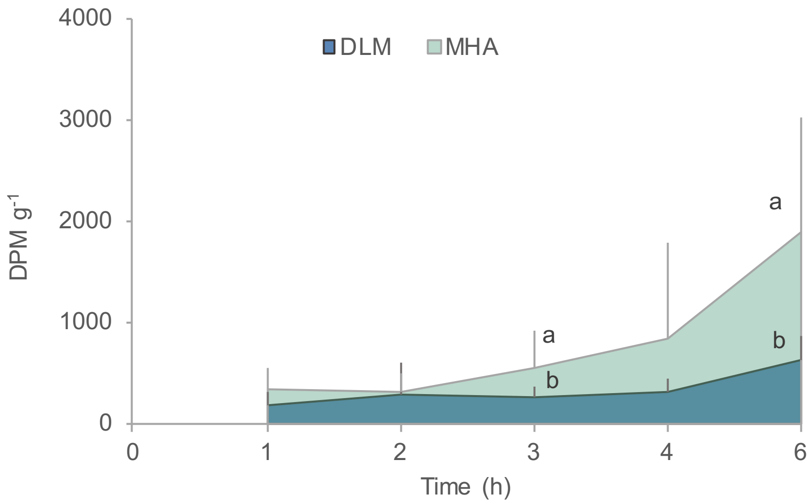

Figure 5.4 Radioactivity (DPM g^{-1} of fish) in the *Incubation Water* compartment at 1, 2, 3, 4 and 6 hours after tube-feeding experimental diets labelled with ^{14}C-DL-Met or ^{14}C-MHA. Values are presented as means ± standard deviation ($n = 6 - 7$ fish for each diet and incubation period). Different letters at the same time-point indicate significant differences ($p < 0.05$) between diets.

The presence of ^{14}C-DL-Met in the *Residual Free* fraction presented a peak at 2 h after diet ingestion (Figure 5.5C), while for ^{14}C-MHA a linear increase was observed during the whole incubation period ($p < 0.05$; Table 5.5). The amount of tracer found in the *Residual Free* fraction of DLM fed fish was significantly higher than in fish fed the MHA diet ($p < 0.05$; Figure 5.5C) at 3 h after diet ingestion. Similar to the *Residual Free* fraction, the amount of tracer found in the *Muscle Free* fraction (Figure 5.5D) of MHA fed fish exhibited a linear increase with time ($p < 0.05$; Table 5.5), while for the ^{14}C-DL-Met a peak was found 2 h after diet ingestion. The amount of ^{14}C-DL-Met found in *Muscle Free* fraction was significantly higher than that of ^{14}C-MHA ($p < 0.05$) at 2 and 3 h after diet ingestion. The area under the curve for the *Residual* and *Muscle Free* fractions were 1.3 and 1.9-fold higher in DLM fed fish than in fish fed MHA diet, respectively, indicating a higher bioavailability of the methionine source in the fish fed the DLM diet (Table 5.5).

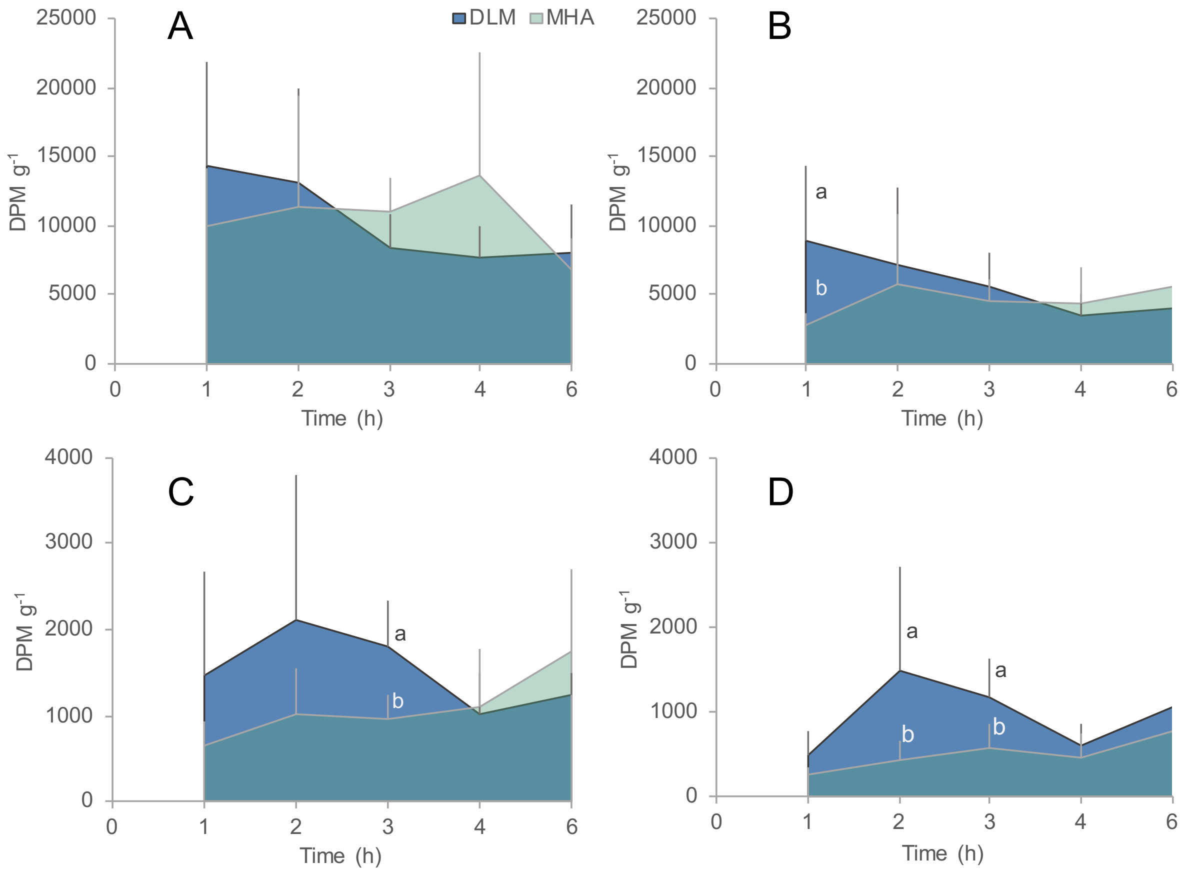

Figure 5.5 Radioactivity (DPM g^{-1} of tissue) in the *Viscera* (**A**), *Liver* (**B**), *Residual* (**C**) and *Muscle* (**D**) *Free* fractions at 1, 2, 3, 4 and 6 hours after tube-feeding experimental diets labelled with ^{14}C-DL-Met or ^{14}C-MHA. Values are presented as means ± standard deviation ($n = 6 – 7$ fish for each diet and incubation period). Different letters at the same time-point indicate significant differences ($p < 0.05$) between diets.

At the end of the incubation period (6 h) a significantly higher amount of tracer was determined in the *Viscera* and *Muscle Protein* fractions from DLM fed fish (Figure 5.6; $p <$ 0.05) No significant differences between treatments were detected in *Liver* and *Residual Protein* fractions ($p > 0.05$). Regarding the *Others* fraction (lipids and other metabolites), there were no significant differences between treatments in all compartments ($p > 0.05$). The amount of tracer present in the *Liver Others* fraction was the lowest (333 and 222 DPM for fish fed the DLM and MHA diets, respectively), and the highest in the *Residual Others* fraction (2858 and 3037 for DLM and MHA fed fish, respectively).

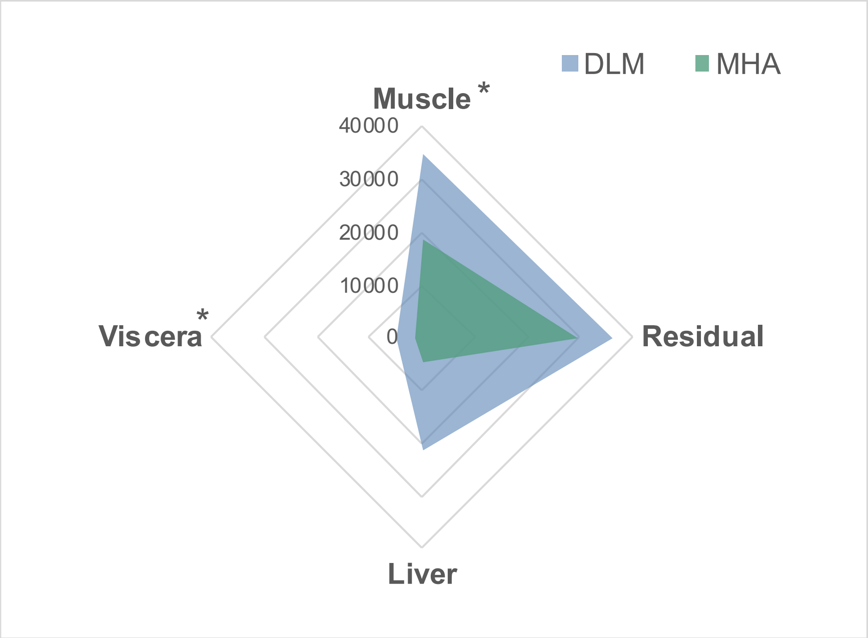

Figure 5.6 Radioactivity (DPM) in the *Viscera*, *Liver*, *Residual* and *Muscle Protein* fractions at 6 hours after tube-feeding experimental diets labelled with ^{14}C-DL-Met or ^{14}C-MHA. Values are presented as means (n = 6 – 7 fish for each diet). Asterisks denote significant differences ($p < 0.05$) between diets within the same compartment.

5.4. DISCUSSION

The *in vivo* method using radiolabelled tracers (^{14}C-DL-Met and ^{14}C-MHA) was utilised to gain a deeper understanding on how different methionine sources may affect the dietary methionine utilisation by Nile tilapia juveniles. The amount of tracer found in the *Incubation Water* increased with time in both treatments, moreover the analysis of the area under the curve from 1 to 6 h after ingestion, revealed a higher amount of ^{14}C-MHA than of ^{14}C-DL-Met indicating a greater evacuation of unabsorbed MHA and/or higher catabolism of this methionine source compared to DL-Met. The solubility of MHA-Ca in water is higher than that of DL-Met (FEEDAP, 2012), however, since the labelled pellets were placed directly into the fish oesophagus and were not in contact with water, the radioactivity present in the *Incubation Water* was the result of the evacuation of unabsorbed tracer. Similarly, in broiler chicks fed ^{14}C-DL-Met or ^{14}C-MHA a higher amount of tracer was present in the excrements of ^{14}C-MHA fed birds than in excrements of birds fed the ^{14}C-DL-Met (Lingens and Molnar, 1996; Saunderson, 1985). DL-Met and MHA uptake is mediated by sodium-dependent transporters across the brush border membrane (To, et al., 2019; 2020), however, MHA is also partly transported by diffusion (Maenz and Engele-Schaan, 1996b). In rainbow trout, it has been demonstrated that MHA presents a slower intestinal absorption rate than DL-Met (To et al., 2020). This indicates that MHA stays longer in the intestine being more prone to bacterial degradation (Drew et al., 2003) that would lead to a further decrease in the intestinal absorption of MHA and its evacuation. Combined, these two factors may contribute to the increased amount of unabsorbed ^{14}C-MHA relative to ^{14}C-DL-Met in the *Incubation Water*.

In our study, *Viscera* and *Liver Free* fractions of DLM and MHA fed fish presented a similar cumulative bioavailability of ^{14}C-DL-Met and ^{14}C-MHA for the 6 h-period. However, a comparison between the two dietary treatments revealed different patterns of tracer flux in these compartments throughout the *in vivo* trial. Fish fed the DLM diet presented a peak in *Liver Free* fraction 1 h after feeding while no peak was observed during the 6 h time-course for MHA fed fish. The D- isomer of DL-Met and L- and D-isomers of MHA need to be converted to L-methionine to become available to the animal via a two-step process (Dibner, 2003; Dibner and Knight, 1984); however, the L-isomer present in DL-Met is readily available. Therefore, the results from the *in vivo* metabolic trial suggest a faster availability of DL-Met to metabolic processes than of MHA.

In fish fed the DLM diet, the tracer was available to the *Residual Free* fraction (*Free* fractions in head, heart, kidney, bones and fins) faster than in MHA fed fish. Moreover, for the 6 h trial, the cumulative methionine source bioavailability in *Residual* compartment was higher in DLM than in MHA fed fish. Although the liver is the major site of conversion of D-Met and of D- and L-MHA to L-methionine, this process also occurs in the kidney (Dibner, 2003; Dupuis et al., 1989; Saunderson, 1985). This may explain the high amount of tracer found in the *Residual Free* fraction, as this compartment includes the kidney among other tissues. Other studies have reported higher amounts of ^{14}C-DL-Met than of ^{14}C-MHA recovered in the kidney of broilers (Lingens and Molnar, 1996; Saunderson, 1985). In a study where chicken kidney homogenates were incubated with ^{14}C-labelled L-Met, DL-Met or MHA, DL-Met was found to be the substrate most readily converted into the intermediate KMB, revealing that the majority of the KMB produced in the kidney would result from the oxidation of the D- and not the L-isomer of DL-Met (Dupuis et al., 1989). Further studies are necessary to confirm if in fish the kidney plays an important role in the conversion of D- to L-Met.

After being converted, the methionine originating from the different dietary methionine sources is transported to the rest of the body to be utilised by the fish. As a consequence of the faster absorption and hepatic metabolism, 2 h after feeding a higher amount of tracer was observed in the *Muscle Free* fraction of DLM fish, implying that dietary methionine is available earlier for utilisation when fish are fed this source. Also, cumulative methionine bioavailability in *Muscle Free* fraction was higher in DLM than in MHA fed fish. Therefore, one can consider that methionine is readily available for protein synthesis faster and for a longer period in DLM fed fish than in fish fed the MHA diet. In fact, 6 h after feeding, the amount of tracer found in the *Protein* fractions of all compartments of DLM fed fish was higher than in MHA fed fish, with significant differences detected in *Viscera* and *Muscle* compartments. This indicates that DLM fish exhibited a faster and more effective incorporation of methionine into muscle protein. Although the amount of tracer present in the *Liver Protein* fraction of DLM fish was higher than in fish fed the MHA diet, the difference was not significant due to a high variability within treatments. The vital physiological functions of the liver in detoxification, protein synthesis and digestion, constantly producing metabolites, may account for this variability.

In both dietary treatments the amount of tracer retained in the lipid fraction, the major component of the *Others* fraction, was very low. Metabolic studies in gilthead seabream (Rocha, et al., 2016a; 2016b) and Senegalese sole (Navarro-Guillén et al., 2017) using a ^{14}C-amino acid mixture have previously demonstrated that absorbed amino acids were preferentially used for protein synthesis and only a small proportion were converted into lipids.

At the end of the growth trial, the content of hepatic free amino acids and one-carbon metabolites was determined in fish fed control and supplemented diets. The content of total hepatic free amino acids in fish fed the DLM diet was significantly higher than in fish fed the REF or the MHA diets, due to an increase in both indispensable and dispensable amino acids. Since these are not postprandial results, it indicates a higher bioavailability of amino acids for metabolic purposes in Nile tilapia fed diets supplemented with DL-Met. Methionine supplementation caused an increment of free methionine in liver, independently of the source. Methionine dietary supplementation studies in Nile tilapia (Michelato et al., 2018) and Atlantic salmon (*Salmo salar*) (Espe et al., 2008; 2014) were unable to observe a similar effect of dietary supplementation in hepatic methionine levels, possibly due to the fact that these studies report postprandrial results unlike the current study where fish were sampled 24 h after feeding, hence the result of basal metabolism. The increase in free methionine levels in liver indicates a higher availability for protein synthesis, transmethylation reactions and antioxidant defence in fish fed supplemented diets (DLM and MHA diets) compared with fish fed the non-supplemented diet (REF diet). In the current study, DLM and MHA fed fish presented similar levels of hepatic methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), while homocysteine was significantly lower in fish fed the DLM diet in comparison to the other dietary treatments. This suggests that the different methionine sources follow different metabolic pathways. In neonatal pigs, to meet methionine requirements, protein synthesis is favoured over methionine transmethylation and the methionine pool is conserved by increasing homocysteine production and suppressing transsulfuration (Bauchart-Thevret et al., 2009). In the present study, the hepatic free amino acid analysis indicate that fish fed MHA and REF diets probably remethylate homocysteine back to methionine, with the addition of a methyl group from trimethylglycine. On the contrary, DLM fed fish seem to divert methionine to the transsulfuration pathway, resulting in a significantly higher hepatic taurine content in DLM compared to MHA fed fish. Similarly, feeding Atlantic salmon with soy-based diets supplemented with DL-Met also resulted in an increase in the transsulfuration pathway and consequently higher hepatic taurine content (Espe et al., 2008). These results indicate a stimulation of the transsulfuration pathway in fish fed the DLM diet.

DLM and MHA fed fish had a similar intake of dietary methionine, cysteine and taurine. The experimental diets were soy-based, hence low in taurine. In fact, taurine content in all diets was below 0.1 mg g^{-1} diet. Taurine is an end-product of methionine metabolism and of the transsulfuration pathway. Therefore, although methionine and cysteine hepatic levels were similar in fish fed DLM and MHA diets, a higher hepatic taurine content was found in the former, probably due to a higher availability of methionine, as indicated by the metabolic trials. Higher hepatic taurine content is beneficial for the fish as taurine is involved in numerous physiological functions. In fish, taurine plays important roles in bile salt formation (Kim et al., 2007; 2008), lipid digestion (Richard et al., 2017), osmoregulation (Takagi et al., 2006) and antioxidant defence (Coutinho et al., 2017; Li et al., 2016; Martins et al., 2019) and it also increases amino acid retention (Pinto et al., 2010; Richard et al., 2017). In Nile tilapia, it has been demonstrated that dietary taurine supplementation improves growth performance (Al-Feky et al., 2016). In the current work, taurine status in DLM fed fish might have partially contributed to the improvement in growth performance observed in the growth trial. Diet supplementation with DL-Met increased fish body weight when compared to REF and MHA fed fish. Relative to the REF diet, DLM diet produced more 214 g in biomass gain, whereas MHA diet produced only more 124 g (on tank basis). This indicates that on equimolar basis MHA is only 58% as efficient as DL-Met in terms of biomass gain. DL-Met supplementation improved feed conversion and protein efficiency ratios when compared to the basal diet (REF), while MHA fed fish presented intermediate results. These results are in agreement with previous studies in fish (Keembiyehetty and Gatlin, 1995; 1997; Powell et al., 2017; Zhou et al., 2021), demonstrating that DL-Met supplementation improves growth and promotes protein accretion more efficiently in Nile tilapia.

In the metabolic trial, it was established that the distinct methionine sources are utilised differently by Nile tilapia juveniles in the short-term. *Residual* and *Muscle Free* fractions shown higher availability of methionine in DLM than in MHA fed fish and 6 h after feeding a greater amount of tracer in the *Muscle Protein* fraction was found in the former. These differences were also reflected in the long-term. At the end of the experimental period, protein retention in DLM fed fish was higher than in fish fed the MHA diet. Protein and total methionine whole-body content were also higher in fish fed the DLM diet than in fish from the MHA group. The differences found between DLM and MHA fed fish in total methionine content and availability explain the differences in protein content, reinforcing that Nile tilapia ultimately utilise DL-Met more efficiently for protein deposition than MHA.

The augmented protein retention was ultimately reflected in the nitrogen balance. All diets were isonitrogenous and feed intake was similar in all treatments, resulting in similar nitrogen intake amongst treatments. However, DLM fed fish presented the highest nitrogen gain and the lowest nitrogen losses, indicating that these fish were more efficient in retaining nitrogen than MHA fed fish. Similar results have been reported in studies performed with rainbow trout juveniles (Powell et al., 2017), where the relative bioavailability of MHA was compared to DL-Met by dose-response trials regarding growth performance and nutrient retention. Lower nitrogen losses are related to higher protein digestibility and/or lower catabolism, resulting in lower nitrogen release to the environment. The *in vivo* metabolic trial revealed that the amount of tracer present in the *Incubation Water* increased with time and was lower in fish fed the DLM diet than in MHA fed fish. Consequently, in the long-term trial this is reflected in the nitrogen balance, indicating that dietary DL-Met supplementation contributes to a reduction in the environmental impact of Nile tilapia farming.

In conclusion, dietary methionine sources influence methionine absorption and utilisation in Nile tilapia juveniles. The *in vivo* study indicated that DL-Met is more retained than MHA probably due to a faster absorption rate as well as a greater availability of free methionine in the tissues to be utilised by Nile tilapia. Additionally, methionine from the different sources appear to follow distinct metabolic pathways; while methionine from the DL-Met seems to be transsulfurated, methionine from MHA and REF diets is probably remethylated to methionine to maintain the free methionine pool. In the long-term, dietary DL-Met supplementation of soy-based diets improved growth performance and nitrogen retention in Nile tilapia, reducing the environmental impact and contributing towards a more sustainable industry.

5.5. ACKNOWLEDGEMENTS

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Chapter VI

A nutritional strategy to promote gilthead seabream performance under low temperatures

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A nutritional strategy to promote gilthead seabream performance under low temperatures

Abstract

Gilthead seabream (*Sparus aurata*) is vulnerable to low water temperature, which may occur in the Southern Europe and Mediterranean region during Winter. Fish are poikilothermic animals, therefore feed intake, digestion, metabolism and ultimately growth are affected by water temperature. This study aimed to evaluate growth performance, feed utilisation, nutrient apparent digestibility, and nitrogen losses to the environment in gilthead seabream juveniles reared under low temperature (~13°C). Three isolipidic and isoenergetic diets were formulated: a diet similar to a commercial feed (COM) that contained 44% crude protein and 27.5% fishmeal, and two experimental diets with a lower protein content of 42% (ECO and ECOSup). In both ECO diets fishmeal inclusion was reduced (10% in ECO and 7.5% in ECOSup diet) and 15% poultry meal was included. Additionally, the ECOSup diet was supplemented with a mix of feed additives intended to promote fish growth performance and feed intake. The ECO diets presented lower production costs than the COM diet, whilst incorporating more sustainable ingredients. Gilthead seabream juveniles (±154.5 g initial body weight) were randomly assigned to triplicate tanks and fed the diets for 84 days. Fish fed the ECOSup diet attained a similar final body weight than fish fed the COM diet, significantly higher than fish fed the ECO diet. ECOSup fed fish presented significantly higher hepatosomatic index than COM fed fish, most likely due to higher hepatic glycogen reserves. The viscerosomatic index of ECOSup fed fish were significantly lower compared to COM fed fish, which is a positive achievement from a consumer’s point of view. ECOSup diet exhibited similar nutrient digestibility than the COM diet. Moreover, feeding fish with the ECO diets resulted in lower faecal nitrogen losses when compared to COM fed fish. The results suggest that feeding gilthead seabream with an eco-friendly diet with a mix of feed additives such as the ECOSup diet, promoted growth and minimised nitrogen losses to the environment. Nutritional strategies that ultimately promote feed intake and diet utilisation are valuable tools that may help conditioning fish to sustain growth even under low temperatures.

Keywords: Gilthead seabream; *Sparus aurata*; Sustainability; Apparent digestibility; Winter temperature; Feed additives.

6.1. INTRODUCTION

Gilthead seabream (*Sparus aurata*) is the most cultivated and economic relevant marine fish species in Southern Europe and in the Mediterranean region and its global production exceeded 228 000 tonnes in 2018 (FAO, 2020). Since the species is mainly produced in cages (Seginer, 2016), fish are constantly exposed to natural seasonal changes during the grow-out period. Fish are poikilothermic animals, therefore influenced by water temperature, which affects their feed intake, digestion, metabolism and ultimately growth (Jobling, 1994). Growth performance of gilthead seabream juveniles under farming conditions is optimal at 24 – 26°C (Hernández et al., 2003) and this species is particularly vulnerable to low temperatures. During Winter, seabream drastically reduces feed intake and may even stop feeding below 13 °C (Ibarz et al., 2003; Tort et al., 1998). In addition, seabream shows an inability to adapt to cold as they do not resume feeding even when kept at low temperature for prolonged periods and when water temperature rises do not immediately restore normal feed consumption (Ibarz et al., 2007a; 2007b; Tort et al., 2004). This cold-induced fasting, causes significant economic losses in fish production as it can last for several months, resulting in low body weight gain or even weight losses (Ibarz et al., 2010; Tort et al., 1998).

Feeding trials are usually conducted under optimum conditions. A limited number of studies have aimed to optimise fish feed formulations under sub-optimal conditions and even fewer studies have focused on formulating specific diets to promote growth of gilthead seabream at low temperature. In previous studies (Richard et al., 2016; Schrama et al., 2017; Silva et al., 2014), a high-quality diet containing high levels of marine-derived protein sources and nutritional supplements was offered to seabream juveniles through Winter and Spring, under natural temperature (mean values: 13°C in Winter and 18°C in Spring) and photoperiod conditions. The supplemented diet seemed to partially counteract the negative effects of exposure to low temperature on fish growth performance and to improve its nutritional and metabolic status through the seasons. However, the aforementioned experimental diets were neither economically viable nor environmentally sustainable as they relied on the inclusion of high levels of protein (~50% crude protein) and fishmeal (40%).

The economic viability and the environmental sustainability of the Aquaculture industry is closely entangled with the reduction of the dietary protein content as well as in the utilisation of more sustainable ingredients to produce aquafeeds. Although crude protein requirements are dependent upon protein digestibility and amino acid profile, dietary incorporation of 45% crude protein has proven to promote growth in gilthead seabream juveniles of 100-200 g (Lupatsch et al., 2003; Santinha et al., 1996). Protein is the most expensive nutrient in the diets (Rana et al., 2009) and therefore, reducing dietary protein inclusion has a positive economic impact, while may result in lower nitrogen excretion into the environment (Bureau and Hua, 2010; Teodósio et al., 2020 (Chapter II)]. One strategy to increase the Aquaculture sustainability is the replacement of marine-derived ingredients such as fishmeal, by ingredients that are eco-friendly but also highly digestible and able to sustain fish growth. Recent studies have pointed towards the use of poultry meal in seabream diets as a good alternative to fishmeal due to its protein content, digestibility and palatability (Davies et al., 2019; Fontinha et al., 2020; Sabbagh et al., 2019). Since it is a by-product of poultry processing plants and slaughterhouses, poultry meal is an environmentally sustainable and cost-effective alternative to fishmeal (Jedrejek et al., 2016).

Nutritional strategies may help conditioning fish to sustain growth even under adverse conditions. The incorporation of feed additives in aquafeeds offers interesting possibilities in fish nutrition (Encarnação, 2016), and might be added for several purposes. For instance, as a phagostimulant, betaine supplementation has increased feed intake in several fish species, such as European seabass, *Dicentrarchus labrax* (Dias et al., 1997), brown-marbled grouper, *Epinephelus fuscoguttatus* (Lim et al., 2015) and gibel carp, *Carassius gibelio* (Xue and Cui, 2001). Since plant protein sources are taurine-deficient, taurine is also frequently added to plant-based diets due to its role in lipid digestion, bile acid conjugation and antioxidant defence (Salze and Davis, 2015), and as an attractant and feed stimulant (Chatzifotis et al., 2009). Also, diet palatability may be enhanced through the inclusion of krill meal, as showed in Pacific white shrimp, *Litopenaeus vannamei* (Derby et al., 2016) and blue shrimp, *Litopenaeus stylirostris* (Suresh et al., 2011). This ingredient has the advantage of also being an excellent source of marine phospholipids (Saleh et al., 2013a; 2018). Phospholipids, from marine and plant origin, have proven to enhance fish growth by improving lipid digestion and/or absorption (Cahu et al., 2003; Saleh et al., 2013b; Tocher et al., 2008). All these nutritional strategies that may promote feed intake and diet utilisation are valuable when formulating diets to help fish cope under adverse conditions.

The present study aimed to evaluate fish growth performance, feed utilisation, nutrient apparent digestibility, and nitrogen outputs to the environment in fish fed experimental diets with lower protein content and environmentally sustainable. The experimental diets differed in the inclusion of feed additives which were incorporated to stimulate feed intake and enhance weight gain. The main goal of this study is to contribute to the optimisation of economically viable and environmentally sustainable diets that are suitable for gilthead seabream ongrowing production at low temperatures.

6.2. MATERIAL AND METHODS

6.2.1. Experimental diets

Three isolipidic (crude fat: ~17.7% as fed) and isoenergetic (gross energy: ~20.9 MJ kg^{-1} as fed) diets were formulated using practical ingredients (Table 6.1). A high protein diet (COM) was formulated to be similar to a commercial feed used for gilthead seabream juveniles, with fishmeal (27.5%) and soy ingredients (14%) as the main protein sources and a crude protein (CP) content of 44%. The other two diets, ECO and ECOSup, were formulated to reduce dietary fishmeal inclusion and protein content (42% CP). In these diets, poultry meal (15%), soy ingredients (11.5%) and fishmeal (10% for ECO and 7.5% for ECOSup) were used as protein sources. In addition, the ECOSup diet contained a mix of feed additives to potentially promote feed intake and feed utilisation, such as: betaine (1%), krill meal (5%), soy lecithin (1%), macroalgae mix (1%) and L-taurine (0.3%). All diets were supplemented with selected indispensable amino acids (IAA) and mono-calcium phosphate, whenever necessary, to fulfil the nutritional requirements of juvenile gilthead seabream. The ECO and ECOSup formulation costs were 86 and 95% relative to the COM diet. The costs of the experimental diets were calculated by the feed manufacturer, SPAROS Lda. (Olhão, Portugal).

Diets (pellet size 3 mm) were produced at SPAROS Lda. by extrusion by means of a pilot-scale twin-screw extruder (CLEXTRAL BC45; Clextral, France) with a screw diameter of 55.5 mm and temperature ranging from 105°C to 110°C. Upon extrusion, all batches of extruded feeds were dried in a vibrating fluid bed dryer (model DR100; TGC Extrusion, France). Following drying, pellets were allowed to cool at room temperature and subsequently the oil fraction was added under vacuum coating in a Pegasus vacuum mixer (PG-10VCLAB; DINNISEN, The Netherlands). Additionally, to measure the apparent digestibility of the diets by the indirect method, 5 kg of each diet was reground, chromic oxide was incorporated at 1% and the mixtures were dry-pelleted (screen diameter: 4.5 mm), using a steamless pelleting machine (CPM- 300; San Francisco, USA). Throughout the duration of the trial, experimental feeds were stored at room temperature, in a cool and aerated storage room. Proximate composition and amino acid analysis were performed for all experimental diets, as reported in Tables 6.1 and 6.2, respectively.

Table 6.1 Formulation and proximate composition of experimental diets.

Ingredients (%)	**COM**	**ECO**	**ECOSup**
Fishmeal [a]	27.50	10.00	7.50
Fish soluble protein concentrate [b]	2.50	2.50	2.50
Squid meal [c]	2.50	2.50	2.50
Krill meal [d]	2.50	2.50	5.00
Poultry meal [e]		15.00	15.00
Soy protein concentrate [f]	10.00	7.50	7.50
Wheat gluten [g]	4.00	4.00	4.00
Corn gluten [h]	7.50	6.00	6.30
Soybean meal [i]	4.00	4.00	4.00
Wheat meal [j]	16.85	22.65	20.45
Faba beans (low tannins) [k]	6.00	6.00	6.00
Sardine oil [l]	10.22	10.15	9.52
Rapeseed oil [m]	4.38	4.35	4.08
Soy lecithin [n]			1.00
Vitamin and Mineral Premix [o]	1.00	1.00	1.00
Lutavit C35 and E50 [p]	0.05	0.05	0.05
Betaine HCl [q]	0.50	0.50	1.00
Macroalgae mix [r]			1.00
Antioxidant powder [s]	0.20	0.20	0.20
Sodium propionate [t]	0.10	0.10	0.10
Mono-calcium phosphate [u]		0.40	0.40
L-Lysine [v]		0.20	0.20
L-Tryptophan [w]		0.10	0.10
DL-Methionine [x]	0.20	0.30	0.30
L-Taurine [y]			0.30

Proximate composition (% as fed)			
Dry matter	92.9	94.0	94.9
Ash	7.4	6.9	7.2
Crude protein	44.2	42.0	42.0
Crude fat	18.0	17.6	17.6
Total phosphorus	1.0	1.1	1.0
Gross energy (MJ kg^{-1})	20.9	20.9	20.8
CP/GE	21.1	20.1	20.2

CP/GE: crude protein to gross energy ratio.

All values are reported as means of duplicate analysis.

[a] Super Prime: 66.3% crude protein (CP), 11.5% crude fat (CF); Pesquera Diamante, Peru.

[b] CPSP 90: 84% CP, 12% CF; Sopropêche, France.

[c] Super prime without guts: 84% CP, 4.7% CF; Sopropêche, Spain.

[d] Krill meal: 61.1% CP, 17.4% CF; Aker Biomarine, Norway.

[e] Poultry meal 65: 67% CP, 12% CF; Sonac, The Netherlands.

[f] Soycomil P: 63% CP, 8% CF; ADM, The Netherlands.

[g] VITAL: 80% CP, 7.5% CF; Roquette Frères, France.

[h] Corn gluten meal: 61% CP, 6% CF; COPAM, Portugal.

[i] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF; CARGILL, Spain.

[j] Wheat meal: 10% CP, 1.2% CF; Casa Lanchinha, Portugal.

[k] Faba beans low tannins: 28% CP, 1.2% CF; Casa Lanchinha, Portugal.

[l] Sopropêche, France.

[m] J.C. Coimbra Lda., Portugal.

[n] Lecico P700IPM; LECICO GmbH, Germany.

[o] INVIVONSA Portugal AS, Portugal: Vitamins (IU or mg kg^{-1} diet): DL-alpha tocoferol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotin acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate, 7.5 mg; sodium chloride, 400 mg; excipient wheat middling's.

[p] BASF, Germany.

[q] Beta-Key 95%, ORFFA, The Netherlands.

[r] Macroalgae mix: 11% CP, 0.6% CF; Ocean Harvest, Ireland.

[s] Paramega PX, KEMIN EUROPE NV, Belgium.

[t] Disproquímica, Portugal.

[u] MCP: 22% P, 18% Ca, Fosfitalia, Italy.

[v] Biolys: L-lysine sulphate, 54.6% lysine; EVONIK Nutrition & Care GmbH, Germany.

[w] L-Tryptophan: 98%; EVONIK Nutrition & Care GmbH, Germany.

[x] DL-Methionine: 99%; EVONIK Nutrition & Care GmbH, Germany.

[y] L-Taurine: 98%; ORFFA, The Netherlands.

Table 6.2 Amino acid composition of experimental diets.

Amino acids *(mg AA g^{-1} as fed)*	**COM**	**ECO**	**ECOSup**
Arginine	34.2	28.3	30.8
Histidine	9.6	9.6	9.7
Lysine	26.8	24.8	29.0
Threonine	16.6	15.1	15.5
Isoleucine	20.6	18.3	19.1
Leucine	34.1	31.8	28.0
Valine	22.2	19.7	20.9
Methionine	14.5	14.2	12.0
Phenylalanine	23.0	19.3	18.6
Cystine	2.9	3.2	2.3
Tyrosine	18.8	18.3	15.3
Aspartic acid + Asparagine	34.6	32.7	35.1
Glutamic acid + Glutamine	67.8	66.4	66.5
Alanine	23.6	20.2	22.4
Glycine	30.3	23.2	27.7
Proline	30.0	25.8	26.0
Serine	17.4	15.5	15.6
Taurine	2.3	2.3	2.3

All values are reported as mean of duplicate analysis.

6.2.2. Zootechnical trials

Experiments were carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the use of laboratory animals. Animal protocols were performed under Group-C licenses by the Direção Geral de Alimentação e Veterinária, Ministério da Agricultura, Florestas e Desenvolvimento Rural, Portugal.

Gilthead seabream juveniles (*Sparus aurata*) were obtained from Atlantik Fish Lda. (Castro Marim, Portugal) and the experiments were conducted at the Ramalhete Experimental

Research Station of the Centre of Marine Sciences (CCMAR, Faro, Portugal). Upon arrival, fish were adapted to new conditions for about one month in a flow-through system with aeration, during which they were fed a commercial diet. Mean water temperature during the adaptation period was 13.0 ± 1.3°C.

6.2.2.1. Digestibility trial

The apparent digestibility coefficients (ADC) of the dietary components were determined by the indirect method, using 1% chromic oxide as a dietary inert tracer, in nine homogeneous groups of gilthead seabream with a mean body weight of 153.5 ± 0.8 g. Triplicate groups of fish (9 fish per tank) were allocated to cylinder-conical 200 L tanks coupled with faeces collectors. Water average temperature was 14.2 ± 0.8°C, salinity 34.0 ± 0.8 psu and dissolved oxygen in water was 90.3 ± 3.9% of saturation. Fish were allowed to adapt to new conditions for one week before starting faeces collection. During the adaptation period fish were fed by hand to apparent satiety once a day one of the experimental diets and continue to do so throughout the trial. Tanks were thoroughly cleaned to remove any uneaten feed. Before feeding, faeces were collected daily for 12 days, left to settle and water was decanted. Faeces were frozen at –20°C and freeze-dried prior to analysis.

The apparent digestibility coefficients (ADC) of the dietary nutrients and energy were calculated as follows (Maynard et al., 1979):

$$\mathrm{ADC}\ (\%) = 100\ \times \left[1 - \frac{\text{dietary } \mathrm{Cr_2O_3} \text{ level}}{\text{faecal } \mathrm{Cr_2O_3} \text{ level}} \times \frac{\text{faecal nutrient or energy level}}{\text{dietary nutrient or energy level}}\right]$$

ADC of dry matter was calculated as:

$$\mathrm{ADC}\ (\%) = 100\ \times \left[1 - \frac{\text{dietary } \mathrm{Cr_2O_3} \text{ level}}{\text{faecal } \mathrm{Cr_2O_3} \text{ level}}\right]$$

6.2.2.2. Growth trial

Fish were reared in 500 L cylindrical tanks supplied with flow-through aerated seawater (temperature: 13.4 ± 2.1°C; salinity: 34.4 ± 0.8 psu; dissolved oxygen in water above 90% saturation) under natural photoperiod conditions (January to mid-April). Daily water temperature data is presented in Figure 6.1. Homogeneous groups of seabream juveniles with a mean body weight of 154.5 ± 13.8 g were distributed in groups of 4 fish, into nine tanks at an initial density of 8.6 kg m^{-3} (28 fish per tank). Five fish from the initial stock were sampled and stored at –20°C for subsequent analysis of whole-body composition. Each experimental diet was randomly assigned to triplicate tanks and tested for 84 days. Fish were fed by hand to apparent satiety once a day (10h00), except Sundays, avoiding feed losses and apparent feed intake was recorded. Mortality, water oxygen saturation and temperature were monitored daily. To monitor growth and feed utilisation, fish from each tank were bulk weighed under moderate anaesthesia every four weeks.

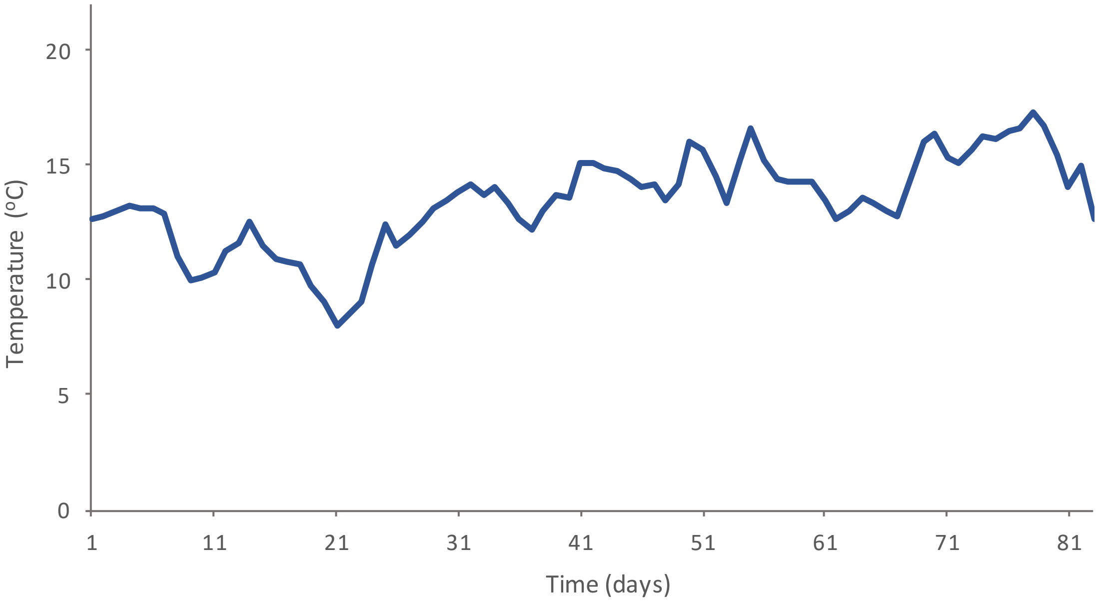

Figure 6.1 Water temperature profile throughout the growth trial.

At the end of the trial, each tank was bulk weighed. Twenty fish from each tank were euthanised with a lethal dose of anaesthetic (1.5 mL L^{-1} 2-phenoxyethanol; Sigma-Aldrich, Spain) and individually weighed. Total length of 10 fish per tank (n = 30 per treatment) was recorded to determine condition factor (K). Five fish from each tank were pooled and stored at –20°C until analysis of whole-body composition (n = 3 pools per treatment). Liver and viscera weight of five fish per tank (n = 15 per treatment) were recorded for calculation of hepatosomatic and viscerosomatic indexes and liver were stored at –20°C until protein and lipid analysis. Fish were fasted for 24 h before initial and final samplings.

6.2.3. Chemical analysis

Chemical analysis followed standard procedures of the Association of Official Analytical Chemists (AOAC, 2006) and were done in duplicates. Before analysis, diets, faeces and pooled whole-body fish were finely ground. Diets and whole-body fish proximal composition was determined as follows: dry matter by drying the samples at 105°C for 24 h and ash content by incineration in a muffle furnace at 550°C for 6 h. Freeze-dried diets, whole-body fish and faeces samples were analysed for crude protein (N x 6.25) using a Leco nitrogen analyser (Model FP-528; Leco Corporation, St. Joseph, USA); crude fat by petroleum ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany); gross energy by combustion in an adiabatic bomb calorimeter (Werke C2000; IKA, Staufen, Germany) calibrated with benzoic acid; phosphorus content by digestion at 230°C in a Kjeldatherm block digestion unit followed by digestion at 75°C in a water bath and absorbance determination at 820 nm (adapted from AFNOR V 04–406) and chromic oxide content was determined according to Bolin et al. (1952), after digestion with perchloric acid. Liver samples were pooled together by replicate tank, freeze-dried and analysed for protein content, as described above, and fat content according to Bligh and Dyer (1959).

Total amino acid profile from both faeces and diets were determined by ultra-high-performance liquid chromatography (UPLC) on a Waters Reversed-Phase Amino Acid Analysis System, using norvaline as an internal standard. Samples were pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using AccQ Tag method (Waters, USA) after acid hydrolysis (HCl 6 M at 116°C for 48 h in nitrogen-flushed glass vials). Amino acids were identified by retention times of standard mixtures (Waters) and pure standards (Sigma-Aldrich). Instrument control, data acquisition and processing were achieved by the use of Waters Empower software.

6.2.4. Calculations

Key performance indicators were calculated as follows:

Weight gain (%) = 100 × wet weight gain × initial biomass^{-1}, where wet weight gain is: final biomass – initial biomass.

Thermal growth coefficient (TGC) = 100 × (FBW$^{1/3}$ – IBW$^{1/3}$) × DD^{-1}, where IBW and FBW are the initial and final body weight, respectively, and DD is the sum of degree.days for the experimental period.

Daily voluntary feed intake (VFI, % day^{-1}) = 100 × apparent feed intake × ABM^{-1} × $days^{-1}$, where ABM is average body mass = (final biomass + initial biomass)/2.

Feed conversion ratio (FCR) = apparent feed intake × wet weight $gain^{-1}$.

Protein efficiency ratio (PER) = wet weight gain × crude protein $intake^{-1}$.

Condition factor (K) = 100 × body weight × total $length^{-3}$.

Hepatosomatic index (HSI %) = 100 × liver weight × body $weight^{-1}$.

Viscerosomatic index (VSI %) = 100 × viscera weight × body $weight^{-1}$.

Nitrogen (N) gain (mg N kg^{-1} day^{-1}) = (final whole-body N content – initial whole-body N content) × ABM^{-1} × $days^{-1}$.

Faecal N loss (mg N kg^{-1} day^{-1}) = [N intake × (100 – N ADC %)] × ABM^{-1} × $days^{-1}$.

Metabolic N loss (mg N kg^{-1} day^{-1}) = N intake – (N gain + faecal N losses).

6.2.5. Statistical analysis

Data are presented as means ± standard deviation. Data expressed as a percentage were arcsine square root transformed previously to the statistical analysis (Ennos, 2012). All data were checked for normal distribution and homogeneity of variances. Differences among dietary treatments were identified by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test at $P < 0.05$ level of significance. Statistical analyses were performed using the open source software R version 4.0.1.

6.3. RESULTS

6.3.1. Digestibility trial

Apparent digestibility coefficients (ADC) of nutrients and energy of experimental diets are presented in Table 6.3. Protein and fat digestibility were high in all diets and were not influenced ($p > 0.05$) by the dietary treatment. Phosphorus ADC values were significantly higher ($p < 0.05$) for ECOSup and COM diets than for ECO diet. Energy digestibility was significantly higher ($p < 0.05$) in diet ECOSup than in ECO diet and not significantly different from the COM diet ($p > 0.05$). Based on these results, calculated values of digestible protein to digestible energy (DP: DE) ratios were 21.9 for the COM diet and 21.2 for both ECO and ECOSup diets (Table 6.3). As for the amino acids, ADC values for the ECOSup and COM diets presented similar values (Table 6.4), with the exception of phenylalanine and tyrosine that presented significantly lower values in the former ($p < 0.05$). Amino acid digestibility of the ECO diet was in general lower than the ECOSup or COM diets.

Table 6.3 Apparent digestibility coefficients (ADC) of nutrients and energy of experimental diets.

ADC (%)	**COM**	**ECO**	**ECOSup**
Dry matter	67.7 ± 1.3	64.9 ± 1.8	69.6 ± 1.9
Protein	89.0 ± 1.5	90.0 ± 3.2	92.3 ± 1.1
Fat	95.4 ± 0.8	94.6 ± 1.4	96.2 ± 0.7
Phosphorus	54.6 ± 1.6^{a}	45.9 ± 1.9^{b}	56.3 ± 2.2^{a}
Energy	85.8 ± 0.9^{ab}	85.5 ± 1.3^{b}	88.1 ± 0.7^{a}
DP/DE ratio	21.9	21.2	21.2

Values are presented as means ± standard deviation ($n = 3$). Different superscripts (a, b) within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences.
Abbreviations: DP, digestible protein; DE, digestible energy.

6.3.2. Growth trial

Twenty-eight and 56 days after being fed the experimental diets, gilthead seabream juveniles presented similar growth performance parameters ($p > 0.05$; data not shown). However, at the end of the growth trial (84 days), seabream fed the ECOSup and the COM diets presented similar ($p > 0.05$) final body weight of 191.4 ± 22.8 g and 193.7 ± 28.8 g, respectively, while ECO fed fish presented a significantly lower ($p < 0.05$) body weight (179.7 ± 29.2 g), from fish fed the COM diet (Table 6.5). It is worth noticing that in this period of low temperatures, all fish were able to increase initial body weight by 25%, 22% and 19% when fed the COM, ECOSup and ECO diets, respectively. Although TGC, VFI, FCR and PER were unaffected by the dietary treatments ($p > 0.05$), ECOSup and COM fed fish presented better performance indicators when compared with fish fed the ECO diet (Table 6.5). Fish condition factor (K) was similar in all dietary treatments, however fish fed the ECOSup diet presented significantly higher hepatosomatic and lower viscerosomatic indexes when compared to fish fed COM diet ($p < 0.05$; Table 6.5). During the trial, survival was high (~98%) and unaffected by the dietary treatments ($p > 0.05$).

Table 6.4 Apparent digestibility coefficients (ADC) of amino acids of experimental diets.

ADC (%)	**COM**	**ECO**	**ECOSup**
Arginine	85.6 ± 0.7^{a}	79.4 ± 1.4^{b}	83.1 ± 2.3^{a}
Histidine	88.8 ± 0.8	86.8 ± 0.5	88.6 ± 1.4
Lysine	88.5 ± 0.5^{ab}	86.2 ± 0.8^{b}	89.4 ± 1.4^{a}
Threonine	87.5 ± 0.4^{a}	83.8 ± 1.2^{b}	85.8 ± 1.4^{ab}
Isoleucine	91.7 ± 0.3^{a}	89.6 ± 0.7^{b}	91.0 ± 1.1^{ab}
Leucine	91.2 ± 0.2	89.4 ± 0.6	89.1 ± 1.4
Valine	91.3 ± 0.3	89.3 ± 0.6	91.0 ± 1.2
Methionine	91.3 ± 0.5	90.3 ± 0.6	89.3 ± 1.2
Phenylalanine	91.9 ± 0.3^{a}	88.8 ± 0.7^{b}	89.6 ± 1.4^{b}
Cystine	88.6 ± 0.7	88.5 ± 0.8	85.9 ± 2.2
Tyrosine	93.6 ± 0.3^{a}	92.7 ± 0.6^{ab}	92.1 ± 0.9^{b}
Aspartic Acid + Asparagine	74.8 ± 1.5	71.3 ± 2.0	75.5 ± 3.4
Glutamic acid + Glutamine	90.5 ± 0.5^{a}	87.6 ± 0.3^{b}	88.8 ± 1.3^{ab}
Alanine	91.0 ± 0.2^{a}	88.4 ± 0.6^{b}	90.6 ± 1.1^{a}
Glycine	95.2 ± 0.2^{a}	93.1 ± 0.4^{b}	94.8 ± 0.7^{a}
Proline	91.6 ± 0.3^{a}	88.7 ± 0.8^{b}	89.7 ± 1.2^{ab}
Serine	86.1 ± 0.3	83.1 ± 1.2	84.6 ± 2.2
Taurine	85.2 ± 0.7	85.1 ± 1.5	87.3 ± 1.3

Values are presented as means ± standard deviation (n = 3). Different superscripts (a, b) within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences.

Table 6.5 Growth performance and somatic indexes of gilthead seabream juveniles fed the experimental diets for 84 days.

	COM	**ECO**	**ECOSup**
FBW (g)	193.7 ± 28.8^{a}	179.7 ± 29.2^{b}	191.4 ± 22.8^{ab}
WG (%)	24.6 ± 3.0	18.5 ± 6.6	22.3 ± 3.0
TGC	0.04 ± 0.01	0.02 ± 0.01	0.04 ± 0.01
VFI (% day^{-1})	0.51 ± 0.02	0.44 ± 0.04	0.48 ± 0.04
FCR	1.8 ± 0.1	2.1 ± 0.3	1.9 ± 0.2
PER	1.3 ± 0.1	1.2 ± 0.2	1.3 ± 0.1
K	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
HSI (%)	2.2± 0.3^{b}	2.3 ± 0.4^{ab}	2.5 ± 0.4^{a}
VSI (%)	5.1 ± 0.7^{a}	4.4 ± 1.0^{ab}	4.0 ± 0.6^{b}

Initial body weight = 154.5 ± 13.8 g for all dietary treatments ($n = 252$).

Values are presented as means ± standard deviation ($n = 60$ for FBW; $n = 30$ for K; $n = 15$ for HSI and VSI; $n = 3$ for the remaining parameters). Different superscripts (a, b) within the same row indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences.

Abbreviations: FBW, final body weight; WG, weight gain; TGC, thermal growth coefficient; VFI, daily voluntary feed intake; FCR, feed conversion ratio; PER, protein efficiency ratio; K, condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index.

At the end of the trial, whole-body composition was not significantly affected ($p > 0.05$) by the dietary treatments (Table 6.6). Moisture content was around 65%, protein content was higher than 16.5%, whole-body fat content presented mean values of approximately 12.5%, phosphorus ranged from 0.8 to 0.9% and energy content varied from 7.8 to 8.1 MJ kg^{-1} (all values in wet weight basis). Dietary treatments did not affect ($p > 0.05$) hepatic protein and fat content (Table 6.6). Furthermore, fish nutrient and energy retention were not affected ($p > 0.05$) by the dietary treatments (results not shown).

Table 6.6 Whole-body and liver composition of gilthead seabream juveniles fed the experimental diets for 84 days.

Body composition (% wet weight)	**COM**	**ECO**	**ECOSup**
Moisture	66.0 ± 0.3	65.5 ± 1.5	65.1 ± 1.2
Ash	3.3 ± 0.5	4.0 ± 0.4	4.0 ± 0.8
Protein	16.6 ± 0.4	16.7 ± 0.8	16.5 ± 0.3
Fat	12.3 ± 0.9	12.4 ± 0.7	12.8 ± 0.9
Phosphorus	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Energy (MJ kg^{-1})	7.8 ± 0.1	8.1 ± 0.5	8.1 ± 0.4
Liver composition (% dry weight)			
Protein	35.6 ± 3.0	35.4 ± 5.2	32.1 ± 1.6
Fat	29.1 ± 3.4	21.0 ± 5.3	22.1 ± 4.8

Initial body composition (% wet weight): moisture = 64.9 %; ash = 3.4 %; protein = 16.4 %; fat = 12.9 %; phosphorus = 0.8 %; energy = 8.2 MJ kg^{-1}.

Values are presented as means ± standard deviation ($n = 3$). Absence of superscripts indicates no significant differences ($p > 0.05$) among diets.

6.3.3. Nitrogen balance

Whole-body composition analysis combined with information on ADC of diets allowed the calculation of daily nitrogen balance (Figure 6.2). Daily nitrogen gain and metabolic losses were unaffected ($p > 0.05$) by the dietary treatments. Daily nitrogen gain varied from 72.7 ± 15.4 to 59.2 ± 22.8 mg N kg^{-1} day^{-1} and metabolic losses from 245.2 ± 23.2 to 203.9 ± 24.8 mg N kg^{-1} day^{-1} for fish fed COM and ECO diets, respectively. ECOSup fed fish displayed intermediate values. However, feeding gilthead seabream juveniles with lower protein content diets ECO and ECOSup resulted in a significant reduction in nitrogen faecal losses. Fish fed the COM diet presented N faecal losses of 39.2 ± 1.3 mg N kg^{-1} day^{-1}, while ECO and ECOSup fed fish lost 29.3 ± 2.7 and 24.6 ± 1.9 mg N kg^{-1} $fish^{-1}$ respectively, to the environment.

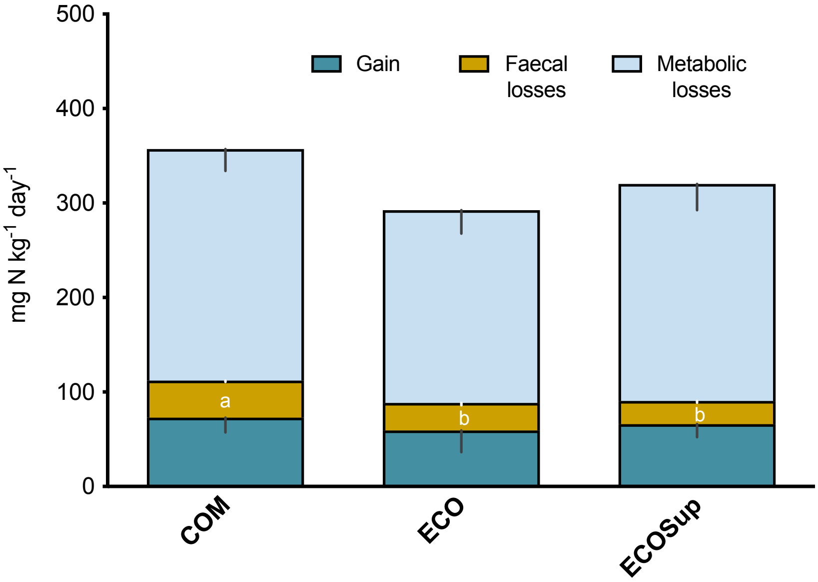

Figure 6.2 Daily nitrogen (N) balance in gilthead seabream juveniles fed the experimental diets for 84 days. Values are presented as means ± standard deviation (n = 3). Different superscripts within bars indicate significant differences ($p < 0.05$) among diets. Absence of superscripts indicates no significant differences ($p > 0.05$).

6.4. DISCUSSION

The present study aimed to evaluate growth performance and diet digestibility in gilthead seabream juveniles fed a diet similar to a commercial feed (COM) and two eco-friendly and less expensive feeds, ECO and ECOSup, under low temperatures (mean water temperature ~13°C). The ECO diets were formulated not only to include environmentally sustainable ingredients in cost-effective feeds, but also to sustain growth under low temperature conditions.

At the end of the growth trial, fish fed the ECOSup diet presented similar growth performance results than fish fed the commercial diet (COM), while ECO fed fish presented significantly lower body weight than fish fed the COM diet. Nutritional studies performed under low water temperature conditions are scarce. However, in a study that evaluated gilthead seabream juveniles (initial weight ± 87 g) growth performance through Winter (mean water temperature ~13°C), individual fish gained 0.08 g per day when fed a control diet (commercial diet, 47% CP as fed basis) and 0.13 g per day when fish were fed a Winter diet (48% CP as fed basis) supplemented with a mix of additives (Silva et al., 2014). Additionally, TGC was improved from 0.01 to 0.02 and FCR from 4.5 to 2.4 when fish were fed the Winter diet. In another study performed at 14°C, after being fed three isonitrogenous (47% CP on dry matter basis) diets that differed in lipid content (14%, 16% and 18%) for 50 days, seabream (initial weight ± 145 g) exhibited similar growth performance indicators for all dietary treatments: mean weight gain was 0.41 g fish^{-1} day^{-1} and FCR ranged from 2.5 to 2.6 (Sánchez-Nuño et al., 2018). In the present study, growth performance parameters were better than previous published experiments under low water temperature: mean weight gain per fish was 0.44 g per day, TGC presented an average value of 0.03 for all dietary treatments and FCR values varied from 1.8 to 2.1. The current results suggest that under low temperature conditions, the ECOSup diet is a valuable alternative to the COM diet in terms of growth and feed utilisation since feeding fish with these diets resulted in similar fish body weight, feed conversion and protein efficiency ratios.

Feeding fish with lower protein and fishmeal content diets (ECO and ECOSup) resulted in similar whole-body composition to fish fed a commercial diet (COM). Whole-body nutrient and energy contents were marginally below or in line with published data for seabream juveniles (Dias et al., 2009; Kissil and Lupatsch, 2004). Fish ingest feed according to their energy demands and at low temperature seabream reduce metabolic activity, lowering their energy requirements (Ibarz et al., 2003). However, lower feed intake may cause a deficit in energy intake that will lead to the utilisation of body energy reserves, preventing fat deposition as perivisceral fat (Ibarz et al., 2005). Fish fed the ECOSup diet presented a significantly lower viscerosomatic index than COM fed fish. No differences were found among treatments concerning whole-body or liver fat content, therefore, it is reasonable to assume that only visceral fat differs between ECOSup and COM fed fish. Fish containing low visceral fat are more likely to be accepted from a consumer’s point of view.

Fish fed the ECOSup diet exhibited a significantly higher hepatosomatic index (HSI) when compared to fish fed the COM diet. Generally, a higher HSI is associated with an increase in fat content in liver (Rueda-Jasso et al., 2004). However, this does not seem to be the case in ECOSup fed fish as there are no significant effects of the dietary treatments in hepatic fat content. Contrary to our results, previous studies have documented fat accumulation in liver of fish subjected to low temperature or thermal shifts (Gallardo et al., 2003; Ibarz et al., 2007a; Ibarz et al., 2005). Furthermore, given the fact that hepatic protein content was also similar among treatments, the differences found in HSI among treatments might be due to changes in energy metabolism and storage of glycogen in the liver. This hypothesis is in agreement with a previous study that used FT-IR spectroscopy to understand how dietary factors affected liver metabolic content in seabream exposed to seasonal temperature variations (Silva et al., 2014). In that study, at the end of Winter, fish fed a commercial diet showed a lower carbohydrate storage than fish fed a Winter feed. The current results suggest that feeding seabream with the

ECOSup diet helps to mitigate the negative effects of low water temperature. Higher energy reserves imply improved fish nutritional status and may be an advantage when optimal growth conditions occur or during periods of low feed intake.

The COM diet had a higher inclusion of fishmeal (27.5%) than the ECO diets. Both ECO diets contained 15% of poultry meal and included fishmeal at 10% (ECO) and 7.5% (ECOSup). Fishmeal has high palatability and is an excellent source of highly digestible protein for most aquatic species (Turchini et al., 2019). It has been considered the gold standard protein source in aquafeeds, especially when feeding carnivorous fish such as the gilthead seabream. However, the dependency of the aquafeed industry on fishmeal has been an environmental concern and a limitation for the continuous growth of the activity; thus, great efforts have been made on finding alternative protein sources (Matos et al., 2017). Due to the European Union (EU) ban in 2001 on the use of terrestrial animal proteins in animal feeds (European Parliament, 2001), research efforts have been focused on the use of vegetable protein sources to replace fishmeal in aquafeeds (Gatlin et al., 2007). However, in 2013 the use of non-ruminant processed animal proteins (PAP) such as poultry meal, has been reinstated in the EU (European Commission, 2013). Poultry meal is made from recycling by-products of slaughterhouses and processing plants, therefore, using poultry meal as a feed ingredient brings value to what would otherwise be perceived as waste material. Furthermore, poultry meal is a highly abundant commodity and is available in markets worldwide (Tacon et al., 2011). Taking all of this into consideration, poultry meal is considered as an environmentally sustainable protein source, with high digestibility and palatability (Oliva-Teles et al., 2015). Previous studies showed that it is possible to replace 50% of fishmeal with poultry meal in seabream diets without compromising growth performance and feed efficiency (Davies et al., 2019; Karapanagiotidis et al., 2019). Other studies in seabream juveniles propose that fishmeal substitution by poultry meal can be up to 83% or even 100% without hindering growth or diet utilisation (Fontinha et al., 2020; Sabbagh et al., 2019). In the present study, no detrimental effects on growth performance and diet utilisation were observed in fish fed the ECOSup diet under low temperature conditions, while fish fed the ECO diet presented lower body weight compared to fish fed the commercial diet (COM). This indicates that poultry meal may be successfully used to replace high dietary levels of fishmeal at low water temperature conditions, if supplemented with an adequate mix of feed additives.

The ECOSup diet contained a mix of feed additives to potentially promote feed intake and utilisation, such as: betaine, taurine, krill meal, soy lecithin and macroalgae mix. Although previous studies showed higher feed consumption by adding betaine (Kolkovski et al., 1997;

Xue et al., 2004) and taurine (Chatzifotis et al., 2009) to the diets, in the present study feed intake was not affected by the inclusion of feed additives. Further investigation is needed to fine-tune the supplementation levels of these compounds as feed additives for the different ongrowing stages as well as rearing conditions. Krill meal and soy lecithin, as sources of phospholipids, have been shown to increase lipid digestion and absorption in seabream larvae (Saleh et al., 2013a; 2013b). In fact, fish fed the ECOSup diet had a lower viscerosomatic index than fish fed the COM diet, suggesting that the supplementation of ECOSup diet resulted in a lower fat deposition due to better utilisation of the dietary lipids. Although the improvement in growth performance and nutrient digestibility cannot be explained by a single additive, the combination of these compounds positively affected gilthead seabream fed the ECOSup diet.

Evaluation of feed digestibility is crucial to maximise nutrient utilisation and minimise aquaculture environmental impact when formulating new diets. In general, seabream fed the experimental diets revealed a high capability to digest nutrients and energy. Digestibility data obtained in the current study is within the range of values reported for seabream fed diets with similar fishmeal and plant protein inclusion levels (Aragão et al., 2020; Dias et al., 2009). The ECOSup diet presented the highest digestibility values for all nutrients, although only differing significantly from the ECO diet for phosphorus and energy. Regarding amino acid digestibility, the ECOSup diet showed results similar to the COM diet, significantly higher than the ones obtained for the ECO diet for most of the amino acids. The higher digestibility of the ECOSup diet implies a greater availability of energy and nutrients to the fish. This is likely to be responsible for the improved growth performance observed in the feeding trial by gilthead seabream fed the ECOSup diet when compared to the ECO fed fish, reinforcing the positive effects of the mix of feed additives to a low protein and low fishmeal diet.

Nitrogen balance calculations for fish fed the different diets revealed that nitrogen gain and metabolic losses were unaffected by the dietary treatments. However, fish fed the lower protein diets ECOSup and ECO, presented significantly lower nitrogen faecal losses than fish fed the COM diet. Fish fed the ECOSup diet lost 7.7% of nitrogen via faeces, a low value especially compared with fish fed the COM diet that lost 11% of the nitrogen intake. Although no significant differences were observed in protein digestibility among treatments, the ECOSup diet presented the highest protein digestibility, which explains lower nitrogen faecal losses and therefore, the release of lower amounts of nitrogen into the aquatic environment. It is vital that environmentally sustainable diets are at optimal requirements for the fish and incorporate highly digestible ingredients so that nutrients losses are reduced (Matos et al., 2017). The reduction of the dietary protein content combined with a higher protein digestibility causes a major positive impact in the reduction of nitrogen release in the environment. The present results support the fact that changing from a COM diet to lower protein diets such as the ECO diets, will help the aquaculture sector to achieve long- term environmental sustainability. Formulating diets that reduce the inclusion of dietary protein as well as fishmeal by replacing it with more sustainable and economic ingredients, is a viable strategy to lower aquaculture production costs and minimise its environmental impact. The reduction of the protein content in aquafeeds has a major impact in the economic viability of the sector. Protein is the major cost associated with feeds and feeds are the main costs of production. The ECO diets presented a lower production cost compared to the COM diet; the ECO diet was 86% of the COM diet while the ECOSup was 95%. This difference between the ECO diets was due to the inclusion of a mix of feed additives in the ECOSup diet that aimed to stimulate feed intake and enhance weight gain. In fact, the ECOSup diet, but not the ECO, resulted in similar fish growth when compared to the commercial diet COM, while reducing nitrogen faecal losses.

6.5. CONCLUSIONS

This study supports the concept that nutrition is a powerful tool to tailor-made diets that will help fish coping with exposure to low water temperatures. Moreover, supplementing a low protein and fishmeal diet with a mix of feed additives as is the case of the ECOSup diet, promoted growth and minimised nitrogen losses to the environment. The current findings suggest that feeding gilthead seabream juveniles with the ECOSup diet may have a major positive impact in the environmental sustainability of the aquaculture sector, while sustaining growth in a critical period such as Winter.

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Chapter VII

General Discussion

7.1. Reduction of dietary protein content does not compromise fish growth performance

Different strategies may be adopted to reduce dietary protein content while maximising fish growth potential, like using supplemental crystalline amino acids to balance the dietary amino acid profile (**Chapters II** and **V**); or diet supplementation with feed additives (**Chapter VI**).

A major challenge for the aquaculture industry is to find new strategies for precision diet formulation that minimise rising feed cost. This cost is largely determined by dietary protein sources and inclusion levels. The five dietary protein contents tested in **Chapter II**, did not affect growth performance and voluntary feed intake of Nile tilapia juveniles, suggesting that the protein content in the several diets was already within the acceptable range for tilapia growth. Only the highest (36%) and the lowest (28%) dietary protein contents were able to significantly change tilapia feed conversion ratio (FCR). FCR for Nile tilapia found in the literature varies significantly, which is likely due to discrepancies in experimental diets and conditions, such as feed quality (physical and nutritional), feeding regime, fish age and size. Considering fish size and dietary protein sources (mainly plant ingredients), the FCR values in **Chapter II** were lower or within the values found in similar studies (Abdel-Tawwab et al., 2010; El-Saidy and Gaber, 2003; Hernández et al., 2010; Silva et al., 2015). Therefore, **Chapter II** demonstrated that it is possible to reduce dietary protein content to 30% without hindering growth performance in tilapia juveniles.

In **Chapter V**, the effect of the different methionine sources (DL-methionine - DL-Met or methyl calcium bis-methionine hydroxyl analogue - MHA) on growth of Nile tilapia was investigated. Fish fed the diet supplemented with DL-Met showed improved growth performance compared with fish fed a non-supplemented diet, while dietary supplementation with MHA resulted in intermediate values. The impact on fish growth was probably due to the distinct metabolic pathways used for converting methionine, which were quite different between the sources. The results reinforce the importance not only of amino acid supplementation but also of its source, since this may influence fish performance even in balanced diets.

The reduction of the dietary protein content through an adequate supplementation proved its feasibility even under adverse conditions, such as low rearing temperatures (**Chapter VI**). Reducing the protein content in diets for gilthead seabream juveniles from 44 to 42% resulted in similar production outputs when the 42% diet was supplemented with a mix of feed additives. The incorporation of feed additives in aquafeeds has been successfully accomplished in enhancing growth, feed intake and diet utilisation in several fish species (Chatzifotis et al., 2009; Kolkovski et al., 1997; Xue and Cui, 2001; Xue et al., 2004). Moreover, the supplementation increased nutrient digestibility implying a greater availability of energy and nutrients to the fish. Although the improvement in growth performance and nutrient digestibility could not be explained by a single additive, the combination of these compounds positively affected gilthead seabream fed the supplemented diet. This indicates that supplementing a low protein and low fishmeal diet with a mix of feed additives is a valuable alternative to a diet with higher protein and fishmeal content (**Chapter VI**).

Unlike the observed results in seabream juveniles reared under low temperatures (**Chapter VI**), protein efficiency ratio (PER) and protein retention in tilapia juveniles were affected with the decrease in the dietary protein content (**Chapter II**). A dietary protein content of 30% resulted in a significant improvement in PER and protein retention when compared with a higher protein diet (36%), while no significant differences were found in amino acid retention (except phenylalanine). Previous studies have shown the importance of an adequate amino acid supplementation to tilapia diets with low inclusion levels of fishmeal to balance the dietary amino acid profile (Figueiredo-Silva et al., 2015; Furuya et al., 2004; Trosvik et al., 2012). In **Chapter II**, the level of supplementation seems to be adequate in the diet containing 30% protein. These results reinforce that the protein content of diets for Nile tilapia juveniles can be reduced to 30% if an appropriate dietary amino acid supplementation is provided (**Chapter II**).

Attaining a reduction of dietary protein through supplemental crystalline amino acids to balance the amino acid profile (**Chapter II** and **V**), or through supplementation with feed additives (**Chapter VI**), proved to be a viable solution for future diet formulations. Nevertheless, the viability of such reduction needs to consider the ingredients included in the formulation while attending to the fish requirements according to species, size and age, and considering the environmental and rearing conditions.

7.2. Promoting nitrogen efficiency minimises aquaculture environmental impact

Reducing the protein content in aquafeeds ultimately limits the nitrogen output to the environment; however, fish require indispensable amino acids for protein synthesis that enables growth and other metabolic functions, and these amino acids must be provided in the diet. Therefore, the challenge lies on providing the necessary building blocks (*i.e.* amino acids) to grow, while minimising the unavoidable losses through amino acid excretion.

Nitrogen losses are due to low protein digestibility and/or amino acid catabolism. The efficiency of absorption and catabolism of individual amino acids will dictate their relative bioavailability as demonstrated in **Chapters III** and **IV**. For instance, in **Chapter III** it was demonstrated that tryptophan was significantly less absorbed than lysine or methionine in seabream juveniles, independently of the diet formulation. A lower digestibility of this amino acid compared to other indispensable amino acids was already reported in Senegalese sole, *Solea senegalensis* (Costas, 2011), rainbow trout, *Oncorhynchus mykiss* (Dabrowski and Dabrowska, 1981), and white seabream, *Diplodus sargus* (Saavedra et al., 2008a) in comparison to methionine or arginine. In addition, seabream preferentially catabolised the ketogenic amino acid lysine, even when this amino acid was deficient in the diet (**Chapter III**). Nevertheless, lysine catabolism can be diminished if the dietary protein to energy (P/E) ratio is increased (**Chapter IV**). This strategy will spare lysine for growth or other metabolic purposes. The results presented in these chapters demonstrate that the optimisation of diets considering amino acids digestibility and bioavailability will maximise protein retention and consequently will minimise nitrogen losses.

Not only the amino acid nature, but also the source, may influence amino acid availability for fish. Tilapia fed a diet supplemented with DL-Met presented significantly higher nitrogen gain than fish fed a non-supplemented or a MHA supplemented diet (**Chapter V**). In fact, fish fed the DL-Met diet showed higher whole-body protein content and protein retention than fish fed the MHA diet, indicating that the former were more efficient in retaining nitrogen. At the end of the growth trial, a time-course metabolic trial was performed using radiolabelled DL-Met and MHA. The *in vivo* metabolic trial revealed that the amount of radiolabelled tracer present in the incubation water, as a result of evacuation and/or catabolism, increased with time and was lower in fish fed the DL-Met diet than in MHA fed fish. Consequently, in the long-term this pattern was reflected in the nitrogen balance, indicating that the methionine source used in diet formulation will influence the environmental impact of fish farming.

The evaluation of diet digestibility is crucial to maximise feed utilisation and reduce nutrient outputs. In **Chapter VI**, the digestibility of a higher protein diet (44%: COM) was compared with two diets with lower protein content (42%: ECO and ECOSup). The low protein diet supplemented with feed additives (ECOSup) presented the highest digestibility for all nutrients. Nitrogen balance of seabream fed low protein diets revealed lower nitrogen faecal losses compared with fish fed the higher protein diet, while nitrogen gain and metabolic losses were similar for all dietary treatments. The reduction of the dietary protein content combined with a higher protein digestibility caused a major impact in decreasing nitrogen losses to the environment (**Chapter VI**).

In **Chapter II**, tilapia fed the high-protein diet (36%) were less efficient in the utilisation of dietary protein than fish fed a diet containing 30% protein. Feed intake was unaffected by the dietary protein level, therefore fish fed the higher protein diets ingested a higher amount of protein. However, this was not translated into improved growth or protein utilisation. Metabolic trials corroborate these findings since fish fed the high-protein diet retained as much protein in the muscle as fish fed the lower protein diets and had a tendency for an increased amino acid catabolism. Amino acid catabolism in fish results in ammonia production and subsequent excretion to the surrounding environment (Ip et al., 2001). Therefore, the tendency for an increased amino acid catabolism found in fish fed the 36% diet is likely responsible for higher ammonia excretion. In fact, fish fed the high-protein diet exhibited similar nitrogen gain, but higher nitrogen losses compared with fish fed lower protein diets (30% and 28%). Previously, it was suggested that dietary excess protein that was not utilised for growth might have been used for energy by tilapia (Abdel-Tawwab et al., 2010). Results from **Chapter II** reinforce the idea and indicate that excessive dietary protein content ultimately results on the use of amino acids for energy and consequently on higher environmental impacts due to increased nitrogen outputs.

The reduction of the environmental impact of aquaculture is closely associated with an efficient use of dietary protein. In **Chapter II**, it was shown that amino acid supplementation of a lower protein diet improved protein utilisation and decreased amino acid catabolism, leading to lower nitrogen losses compared with fish fed the high-protein diet. In **Chapter V** it was demonstrated that fish fed a diet supplemented with DL-Met were more efficient in the retention of protein than fish fed the MHA supplemented diet. Furthermore, the incorporation of a mix of feed additives in a lower protein diet resulted in improved protein digestibility and lower faecal nitrogen losses, as presented in **Chapter VI**. All the above approaches lead to an improvement in nitrogen efficiency and to the consequent decrease in nitrogen outputs to the aquatic environment.

7.3. The metabolic fate of dietary amino acids is mainly determined by their nature

The metabolic fate of amino acids and their availability in fish tissues to be incorporated into proteins for growth or for other metabolic functions is influenced by numerous factors such as diet digestibility, dietary protein and lipid content, amino acid profile and P/E ratios

(Halver, Hardy, 2003). In **Chapter III**, the metabolic fate of lysine, tryptophan or methionine in seabream fed diets with distinct protein and/or lipid content (44P21L, 44P18L, 40P21L and 40P18L) was mainly affected by the nature of the amino acids rather than the diet formulations. Similar results were obtained in **Chapter IV** in seabream fed diets differing in the inclusion of lipids and with distinct P/E ratios (LowP/E and HighP/E diets). Nevertheless, dietary P/E ratios influenced amino acid utilisation and metabolism (**Chapter IV**).

In **Chapter III**, the low digestibility of tryptophan observed in preceding studies was confirmed (Costas, 2011; Dabrowski and Dabrowska, 1981; Saavedra et al., 2008a). Independently of the diet, this amino acid was significantly more evacuated than lysine or methionine, probably due to a lower affinity to intestinal transporters and/or interactions with other amino acids. Although in **Chapter III** diet formulations with different protein and/or lipid levels did not affect the evacuation of the selected amino acids, in **Chapter IV** the decrease in the P/E ratio by an increase in dietary lipid content led to a decrease in amino acid evacuation in gilthead seabream juveniles.

Amino acid retention in the liver was influenced by the levels of protein (**Chapter III**) and not lipids (**Chapter IV**) in the diets. In **Chapter III**, fish fed the low protein diet (40%) retained more amino acids in the liver than fish fed the high protein diets (44%), suggesting that when fish are fed with less protein, amino acids may be provisionally retained in the liver to ensure their availability for metabolic purposes. The results from **Chapter IV** established that protein synthesis is occurring in the liver since amino acids are being mostly incorporated into hepatic proteins within 18 h after feeding and not only temporarily available in the free pool.

The retention of protein-bound amino acids in the muscle was neither affected by the dietary P/E ratios nor the nature of the amino acids (**Chapter IV**). However, in **Chapters III** and **IV**, total amino acid retention in the muscle, *i.e.* the sum of protein-bound and free amino acids, was determined by the amino acid nature, being methionine the most retained amino acid in this tissue. Similarly, in Senegalese sole larvae and juveniles and in white seabream larvae, most of the methionine was recovered in the muscle (Costas, 2011; Pinto et al., 2013; Rønnestad et al., 2000; Saavedra et al., 2008a). Additionally, a lower dietary P/E ratio resulted in a higher retention of lysine and methionine in the muscle free pool, while the retention of the overall amino acid mixture remained relatively unaltered (**Chapter IV**). Methionine was the most retained amino acid in the muscle free pool, reinforcing its essential role in several metabolic functions beyond protein synthesis. In fish fed LowP/E and HighP/E diets, lysine retention in the muscle was similar to protein, confirming that lysine is a good indicator of muscle accretion. The combination of these results indicate that dietary P/E ratios affect amino acid utilisation and may favour the retention of indispensable over dispensable amino acids in the muscle.

The interaction between the nature of the distinct amino acids and the dietary P/E ratios affects amino acid catabolism. In **Chapters III** and **IV**, independently of the dietary formulation, amino acid catabolism in seabream was primarily ketogenic considering that lysine was the most catabolised amino acid even if deficient in the diet (**Chapter III**). While protein and methionine catabolism remained stable in fish fed diets differing in the inclusion of lipids and P/E ratios, lysine catabolism decreased with an increase in the dietary P/E ratio (**Chapter IV**). It appears that a higher availability of free lysine in the muscle of fish fed the LowP/E diet resulted in increased catabolism. While fish fed the LowP/E diet presented values as high as the ones for protein, fish fed the HighP/E diet drastically reduced lysine catabolism. Fish are able to selectively retain and catabolise distinct amino acids (Aragão et al., 2004; Saavedra et al., 2008a, 2008b). Results from **Chapter IV** suggest that although fish fed both diets were equally using amino acids for energy, the dietary P/E ratios influenced which amino acids were catabolised. An increase in the P/E ratio in seabream diets reduced lysine catabolism and may channel other amino acids (*e.g.* dispensable amino acids) for energy, increasing lysine availability for growth or other metabolic functions.

The bioavailability and metabolism of individual amino acids should be considered when formulating diets to improve feed utilisation and fish performance. In seabream juveniles, methionine is preferentially retained in the muscle while lysine is catabolised. The **Chapters III** and **IV** demonstrated that the metabolic fate of amino acids is mainly determined by their nature.

7.4. Dietary methionine influences fish metabolism and growth

A balanced dietary amino acid profile is required to increase amino acid retention and improve growth and nitrogen utilisation (Aragão et al., 2004). Fish diets that incorporate large amounts of plant ingredients may be limited in some indispensable amino acids, especially lysine and methionine, and as seen in **Chapter II**, need supplementation with crystalline amino acids to fulfil fish requirements. In **Chapters III** and **IV** it was demonstrated that factors such as the nature of amino acids or dietary P/E ratios, may influence amino acid absorption and metabolism, and ultimately fish growth performance.

In **Chapter V**, fish body weight, FCR, PER, as well as protein and energy retention, were improved with methionine supplementation, demonstrating the importance of supplementing diets with this indispensable amino acid. Nevertheless, methionine utilisation in Nile tilapia was conditioned by the dietary source. Diets supplemented with synthetic forms of methionine, DL-Met and MHA, resulted in substantial differences in terms of growth, methionine metabolism and nitrogen balance. During the 6 h *in vivo* metabolic trial, dissimilarities between the two sources were revealed in absorption and availability of free methionine in fish tissues. Not only MHA was more evacuated than DL-Met, but also DL-Met became available earlier to be utilised by Nile tilapia than MHA, most likely due to a faster and more efficient absorption rate as also demonstrated in rainbow trout (To et al., 2020). Furthermore, the cumulative availability of free methionine in the muscle was higher in fish fed a diet supplemented with DL-Met than if supplemented with MHA. In fact, 6 h after ingestion, protein-bound methionine in muscle was higher in fish fed the DL-Met diet than in fish fed the MHA diet. These results suggest that DL-Met fed fish exhibit a faster and more effective incorporation of methionine into muscle protein, leading to a more efficient retention of nitrogen that contributes to the improved growth and diet utilisation observed during the growth trial.

After 18 h of ingesting diets with distinct protein and/or lipid levels, most of the methionine was retained in the muscle of gilthead seabream juveniles, even when diets exhibited a deficit in sulphur amino acids (methionine + cysteine) (**Chapters III** and **IV**). The fact that methionine is preferentially retained in this tissue, independently of the diet formulation, suggests the importance of this amino acid to be available in muscle for growth and other metabolic functions. In fact, in **Chapter IV**, it was possible to demonstrate that the higher retention of methionine was due to its presence in the free pool, reinforcing the fundamental role of methionine in several metabolic functions other than protein synthesis and highlighting how overall growth and metabolism are affected by this amino acid.

7.5. Nutritional strategies improve fish robustness

Fish growth may be the ultimate objective of a fish farm but for fish to reach their full growth potential it is essential that they are able to withstand adverse conditions that may occur during the production cycle. Stressful conditions such as high rearing densities, handling procedures or low temperatures, may hinder feed intake and utilisation. Nutritional approaches may help conditioning fish to sustain growth, improving their nutritional status and help fish cope with unfavourable situations.

In gilthead seabream juveniles reared at low water temperatures, the incorporation of a mix of feed additives in a low protein diet (ECOSup) ensured similar growth performance compared with fish fed a commercial diet (COM) with higher inclusion of protein (**Chapter VI**). Additionally, fish fed the ECOSup diet exhibited a significantly higher hepatosomatic index (HSI) than COM fed fish. Considering that fish fed both diets had a similar body weight as well as hepatic protein and lipid content, the augmented HSI is most likely caused by differences in energy metabolism and storage of glycogen in the liver. This hypothesis is in agreement with a previous study that used FT-IR spectroscopy to understand how dietary factors affected liver metabolic content in seabream exposed to seasonal temperature variations (Silva et al., 2014). In that study, at the end of Winter, fish fed a commercial diet showed a lower carbohydrate storage than fish fed a diet supplemented with feed additives. Results from **Chapter VI** suggest that feeding seabream with the low protein diet supplemented with a mix of additives helps to mitigate the negative effects of low water temperature since higher energy reserves imply improved fish nutritional status and may be an advantage when optimal growth conditions occur or during periods of low feed intake.

In **Chapter V**, the hepatic free amino acid content was increased in Nile tilapia fed a diet supplemented with DL-Met, due to the increment of both indispensable and dispensable amino acids. Such an increment implies a higher availability of amino acids to be used in essential metabolic processes in fish fed the DL-Met diet than in fish fed a non-supplemented or a MHA supplemented diet. Independently of the source, the supplementation of methionine in diets for tilapia resulted in an increase of free methionine in the liver, indicating a higher availability of methionine for protein synthesis, transmethylation reactions and antioxidant defence in these fish compared with fish fed the non-supplemented diet. Nevertheless, the analysis of hepatic one-carbon metabolites revealed some significant discrepancies as a result of the dietary supplementation, suggesting that the different methionine sources follow different metabolic pathways. While fish fed the MHA diet probably remethylate homocysteine back to methionine, with the addition of a methyl group from trimethylglycine, fish fed the DL-Met diet seem to divert methionine to the transsulfuration pathway, resulting in a significantly higher hepatic taurine content in DL-Met compared to MHA fed fish. Higher hepatic taurine content is beneficial for the fish since taurine plays important roles in bile salt formation (Kim, et al., 2007, 2008), lipid digestion (Richard et al., 2017), osmoregulation (Takagi et al., 2006) and antioxidant defence (Coutinho et al., 2017; Li et al., 2016; Martins et al., 2019) and it also increases amino acid retention (Pinto et al., 2010; Richard et al., 2017). In Nile tilapia, it has been demonstrated that dietary taurine supplementation improves growth performance (Al-Feky et al., 2016). In **Chapter V**, taurine status in DL-Met fed fish might have partially contributed to the improved growth performance. Therefore, the supplementation of diets with

DL-Met not only will improve growth but also result in a greater availability of free methionine and taurine. These amino acids have important metabolic roles that may help improve fish robustness.

Methionine is preferentially retained in fish muscle, as seen in **Chapters III** and **IV**. However, results from **Chapter IV** demonstrate that 18 h after diet ingestion, most of the retained methionine in muscle was present in the free pool. Methionine participates in numerous metabolic reactions as a precursor of SAM (S-adenosylmethionine), cysteine, glutathione, taurine, and polyamines (Brosnan and Brosnan, 2006; Wu, 2013). Moreover, this indispensable amino acid has been shown to be involved in the methylation of DNA and protein, cell proliferation and differentiation (Li et al., 2009; Wu, 2009). A higher availability of free methionine is an advantage since it indicates a faster and more effective mobilisation in response to specific metabolic functions of fish.

Tailor-made diets help fish cope with adverse conditions. It is therefore essential to properly address nutritional aspects to formulate fine-tuned diets that are adapted to different conditions and improve fish robustness, while sustaining growth and maximising feed utilisation.

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Chapter VIII

Conclusions and Future Perspectives

8.1. CONCLUSIONS

The present Thesis suggests the following conclusions:

- The protein content of diets for Nile tilapia juveniles can be reduced to 30% without compromising fish growth performance, while decreasing nitrogen losses to the environment (**Chapter II**).
- Diet formulations should consider amino acids bioavailability and metabolic fate to promote protein retention and enhance fish growth (**Chapters III** and **IV**).
- The metabolic fate of dietary amino acids is mainly determined by their nature. In gilthead seabream methionine is preferentially retained in the muscle while lysine is catabolised (**Chapters III** and **IV**). Nevertheless, lysine catabolism may be reduced by increasing the dietary protein to energy ratio (**Chapter IV**).
- Methionine absorption and utilisation are influenced by the dietary source in Nile tilapia juveniles. DL-methionine supplementation of soy-based diets improves growth performance and nitrogen retention, reducing the environmental impact of tilapia farming (**Chapter V**).
- Assessing dietary effects through tracer studies using *in vivo* models will help maximising protein accretion, as well as formulating targeted diets (**Chapters III**, **IV** and **V**).
- The supplementation of feed additives in diets for gilthead seabream juveniles enables the decrease of protein and fishmeal contents by increasing diet digestibility and lowering nitrogen faecal losses. Additionally, fish growth is ensured even in adverse rearing conditions such as low water temperatures (**Chapter VI**).

This Thesis generated relevant knowledge on various nutritional strategies to promote nitrogen retention and maximise fish growth, contributing to the development of a more sustainable aquaculture.

8.2. FUTURE PERSPECTIVES

This Thesis has demonstrated that not only feed formulation, but also the ingredients, have vast impacts on fish performance and will influence the nitrogen losses to the environment. Given the expected growth of the aquaculture industry as a supplier of nutritious animal protein in a rising population, the use of eco-friendly feeds to reduce the environmental footprint of the sector is part of the solution towards a more sustainable fish production. As showed in Chapter VI, it is possible to optimise diets for a specific condition by including feed additives. Understanding how to tailor diets for aquaculture production is pivotal for the development of a sustainable and environmentally friendly industry.

In addition, further research and investment is needed to include novel ingredients in aquafeeds in a cost-effective way. Rather than a single approach, the development of a more sustainable aquaculture relies on the creation of synergies among different sectors, allowing for the application of a circular economy approach. The concept of a circular model is to reduce waste and pollution, keep products and materials in use and regenerate natural systems.